

SILSOE COLLEGE,  
CRANFIELD INSTITUTE OF TECHNOLOGY,  
THE DEPARTMENT OF FOOD RESEARCH AND TECHNOLOGY

DOCTOR OF PHILOSOPHY

1990

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GELATION AND MELTING OF GELATIN

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March, 1990

TO MY WIFE AND FAMILY

ACKNOWLEDGEMENTS

I am most grateful to my friends and colleagues who have encouraged and enabled me to pursue this research within the Department of Food Research and Technology at Silsoe College, and also to my external collaborators at Unilever Research, Colworth House, in particular Dr. I.T. Norton, Dr. S.B. Ross-Murphy, Dr. J.P. Busnel and Mr. C.R.T. Brown for practical advice and numerous helpful discussions. I also thank Mr. N.J.M. Brindle for his assistance in Fortran programming.

I am particularly indebted to my supervisor Professor E.R. Morris who, throughout all aspects of this work, has been a constant source of encouragement and enthusiasm, always at hand to provide help and assistance whenever required no matter what the problem.

Thanks are also due to my family and my wife for the many ways in which they have helped, both practically and indirectly by just being there when things were not proceeding as planned.

I acknowledge the financial support provided in the form of a co-operative studentship award by the Agricultural and Food Research Council and Unilever Research, and finally, but by no means least, many thanks go out to Mrs. P. Pugh for her patience and skill in both the deciphering and typing of this thesis.

ABSTRACT

Chiroptical, rheological and thermodynamic studies have been undertaken to investigate temperature-induced changes in the molecular organisation of gelatin. From the results obtained, a unified model for gelation and melting has been developed, and tested using Monte Carlo computer simulation.

The temperature at which gelatin gels are formed has a major influence on the properties of the resulting network, with higher curing temperatures conferring increased thermal stability. In particular, gels formed by sequential curing at two different temperatures show biphasic melting. This is explained in terms of a temperature-dependence of helix length within the junction zones of the gel, and quantified by considering end-effects in the thermodynamics of helix stability. Measurements of 'initial slope' kinetics, performed over a broad concentration range, showed first-order kinetics at low gelatin concentrations, while at higher concentrations a second-order process was also evident. The results are interpreted as triple-helix nucleation at metastable 'hairpin turns' in one chain (bringing two chain segments into close proximity) together with a third strand from either the same chain (first order) or a different chain (second order). From simple geometric considerations, the maximum length of intermolecular helices (which contribute to the gel network) is greater than that of 'wasted' intramolecular structures, giving a qualitative explanation of the increased strength of gels formed by precuring at higher temperatures (where only long helices are stable) over those quenched directly to low temperature.



Monte Carlo simulation incorporating an initial assumption that helix propagation is rapid and proceeds to geometric limits gave unrealistic helix lengths and simulated melting profiles, and was replaced by the assumption that *cis-trans* isomerisation of peptide bonds is the controlling factor in helix propagation. Using the latter assumption, most aspects of the observed behaviour were successfully reproduced using program variables set within realistic ranges or, where possible, fixed at experimentally-determined values. In particular, the co-operativity of the simulated melting process was critically dependent on the value of a parameter  $x$  (the number of triplet units within each helix incapable of participating in bonding, due to end-effects), with a value of  $x = 1$  giving the best fits with experiment (consistent with accepted bonding patterns for the collagen triple helix). Other key parameters were the midpoint temperature for melting of the parent collagen, which gave best agreement when set at 37-38°C, and the proportion of *cis* peptide residues present in disordered gelatin chains, with an optimum lower limit of 0.15.

Using these values, the simulation reproduced, with excellent precision, the helix fraction and melting profile of gels formed over a wide range of quench temperatures, and gave an acceptable approximation to the form of reaction progress curves obtained for helix formation. The biphasic melting of samples held at intermediate temperature before final quenching was also modelled realistically.

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## CHAPTER 1: GENERAL INTRODUCTION TO PROTEIN STRUCTURE AND CONFORMATION

### 1.1. INTRODUCTION TO PROTEINS

Proteins are the most abundant macromolecules in most cells, comprising some 50% or more of the dry weight. They are the most diverse of biological molecules, functioning as enzymes, hormones, transport agents and structural components. Proteins can be divided into two classes on the basis of their compositions: simple proteins are composed entirely of amino acids whereas conjugated proteins yield not only amino acids on hydrolysis, but also other organic or inorganic components such as lipids, nucleic acids, carbohydrates, phosphate groups, iron and zinc.

The molecular weights of proteins can be measured by various physico-chemical techniques and most have values in the range 12 - 1000 kD or greater.

Generalizations about protein size and function are not possible however, since many proteins have a similar type of function but completely different molecular weights.

Proteins can also be categorised according to their physical characteristics. There are two major groups, globular and fibrous proteins. Globular proteins are



water-soluble and diffuse readily; the protein chain is folded into a specific, compact spherical or globular shape allowing the protein a mobile or dynamic function. Examples of globular proteins include nearly all enzymes, and blood proteins such as haemoglobin and serum albumin, having a transport function. Fibrous proteins are water-insoluble and physically strong; the protein chains are arranged in extended, often helical form along a single axis. Examples of this class of proteins are a keratin, the major constituent of hair, feathers, nails and skin, and collagen, the major constituent of tendons.

## 1.2. LEVELS OF STRUCTURE IN PROTEINS

In common with other biopolymers, the structure of proteins can be conveniently broken down into four different levels; primary, secondary, tertiary and quaternary structure.

The complete covalent structure of a biopolymer chain is called the primary ( $1^\circ$ ) structure and is defined by the composition and sequence of the constituent monomers. For proteins the individual monomeric residues are amino acids, which are linked through the peptide bond.

Secondary (2°) structure can be defined as the local spatial organisation of the polymer backbone without consideration of the side chain conformations. The side chain conformations do however determine the type of secondary structure attained through favourable molecular interactions. Typical examples of protein secondary structure include  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn and the collagen triple helix.

The tertiary (3°) structure of a protein is defined as the packing of the secondary structural elements within a chain into a three dimensional structure of one, effectively indivisible, unit. The tertiary structure is generally characteristic of a particular protein, although closely related proteins sometimes have very similar tertiary structures.

Finally, quaternary (4°) structure is the assembly of the tertiary structural elements into an oligomeric form which may contain multiple copies of a single type of chain, or two or more chemically distinct chains.

### 1.3. AMINO ACIDS

The diversity in size, shape and function of proteins is a consequence of the number, type and sequence of amino acids in the protein chain. Naturally-occurring proteins are composed of up to twenty two different amino acids (Table 1.1.) which can be regarded as the "chemical alphabet" used to form the different "protein words".

Biologically-occurring amino acids have two important structural features in common:-

- 1) They are all  $\alpha$  amino acids (i.e. the amino group is bonded to the carbon next to the carboxyl group).
- 2) With the exception of glycine, which is achiral, all exist in the L-configuration at the chiral  $\alpha$  carbon.

Chirality arises from the ability of asymmetric carbon atoms (i.e. carbon atoms with four different substituent groups) to exist in two isomeric forms which are mirror images of each other. The stereoisomers are chemically identical, except in reactions with other chiral materials and physically identical, except in their interaction with plane polarised light (rotating the plane of polarisation either to the right or to the left). The ability of



amino acids to behave in this manner is of importance in the conformation studies of proteins, as will become evident in later chapters. By convention the L-configuration of amino acids is designated relative to that of L-glyceraldehyde (Fig. 1.1.)

The twenty two biologically-occurring amino acids can be subdivided into five groups according to the covalent structure of their sidechain, R (Table 1.1.). The sidechains can be neutral, hydroxylated, sulphur-containing, acidic or basic. Phenylalanine, tryptophan, tyrosine and histidine are aromatic in character while in proline and hydroxyproline the sidechain is bonded to the  $\alpha$ -amino group producing a cyclic secondary amine (an imino acid). Cysteine can occur in two forms, either as a single cysteine residue or in the form of cystine, in which two cysteine molecules are linked together by a disulphide crosslink formed by oxidation of their thiol groups. The properties of the sidechains, particularly polarity and size, play significant roles in determining protein conformation.

Amino acids contain both basic ( $-\text{NH}_2$ ) and acidic ( $-\text{COOH}$ ) groups, i.e. they are amphoteric. In the crystalline state they exist as dipolar ions (Zwitter ions), while in solution there is an equilibrium between the dipolar ion, the anionic form and the cationic form (Fig. 1.2a). The predominant form



present is dependent on pH, being cationic under strongly acid conditions and anionic under basic conditions. At some intermediate pH however, determined by the pKa's of the relevant protons, the concentration of the dipolar ion becomes a maximum, the concentrations of the cationic and anionic form being equal (Fig. 1.2b). This point is known as the isoelectric point (pI), at which a solution of an amino acid undergoes dramatic changes in various physical properties such as density, conductivity, etc.

The values of the pKa's and pI's for all amino acids are known and are given in Table 1.1.

Obviously the pH of an amino acid solution is critical in determining the solution properties, and the situation becomes more complex when more than one amino acid is present. In proteins, where the amino acids are linked through the peptide bond, there is still only one terminal amino group and one terminal carboxyl group in a polypeptide chain but additional ionizable protons on the sidechains make the titration curves of proteins considerably more complex.

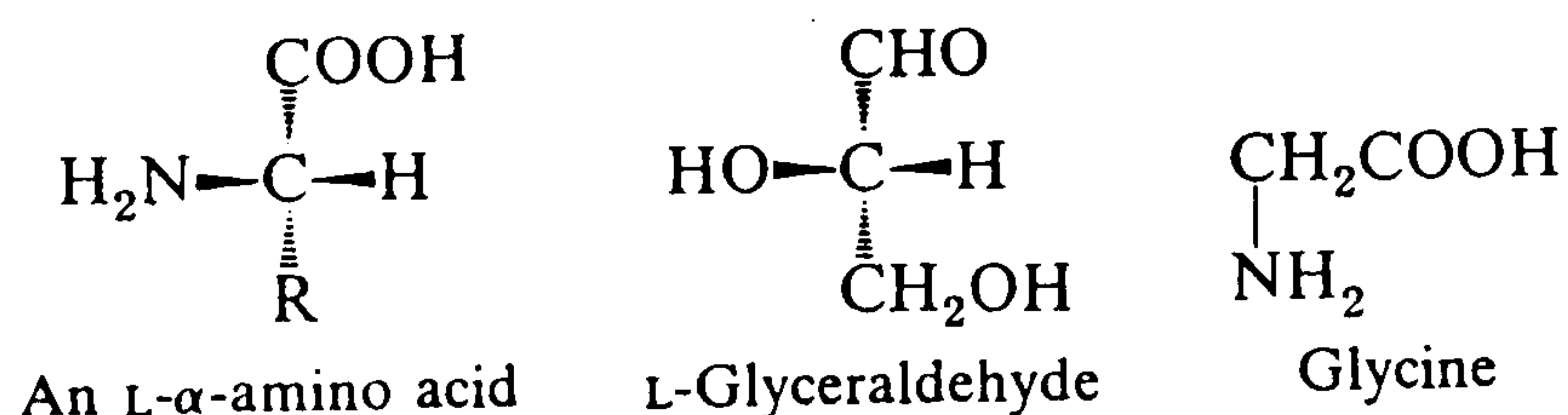
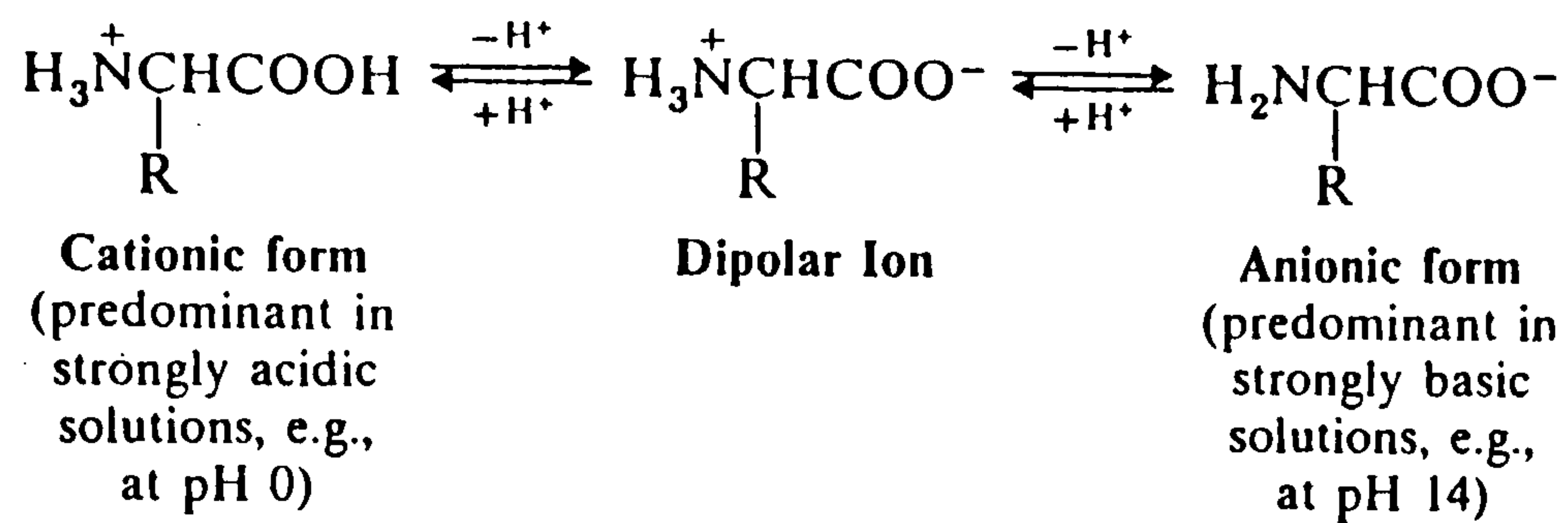


Figure 1.1. The L-configuration of glyceraldehyde and the corresponding L-configuration of an  $\alpha$  amino acid. Also shown is the achiral amino acid, glycine.

a.



b.

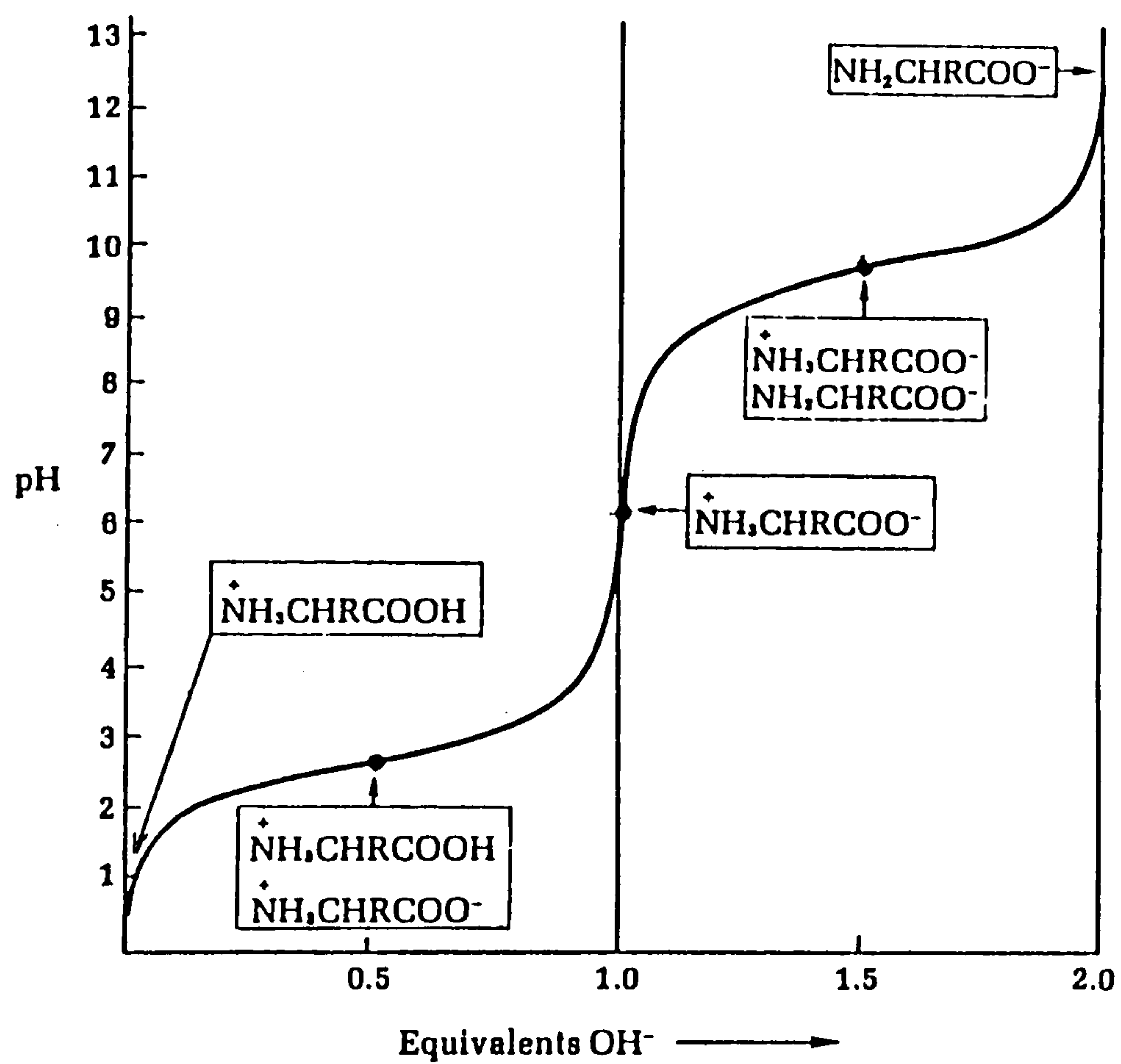
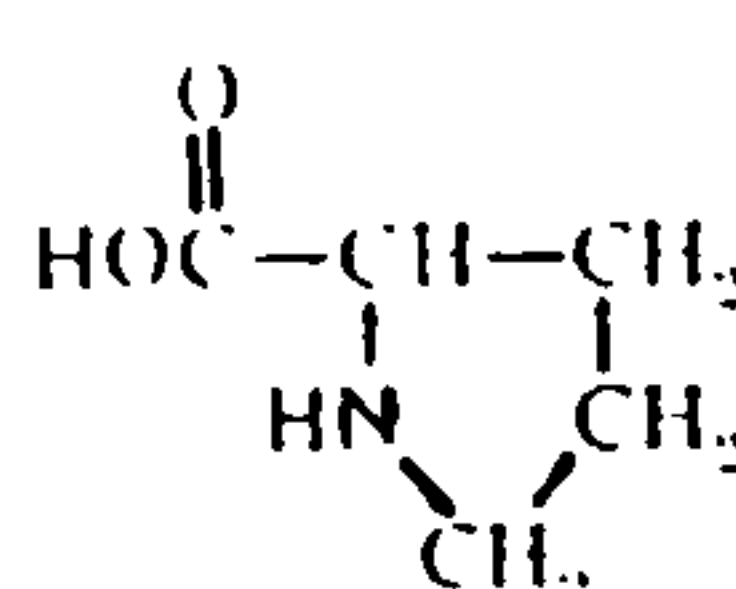
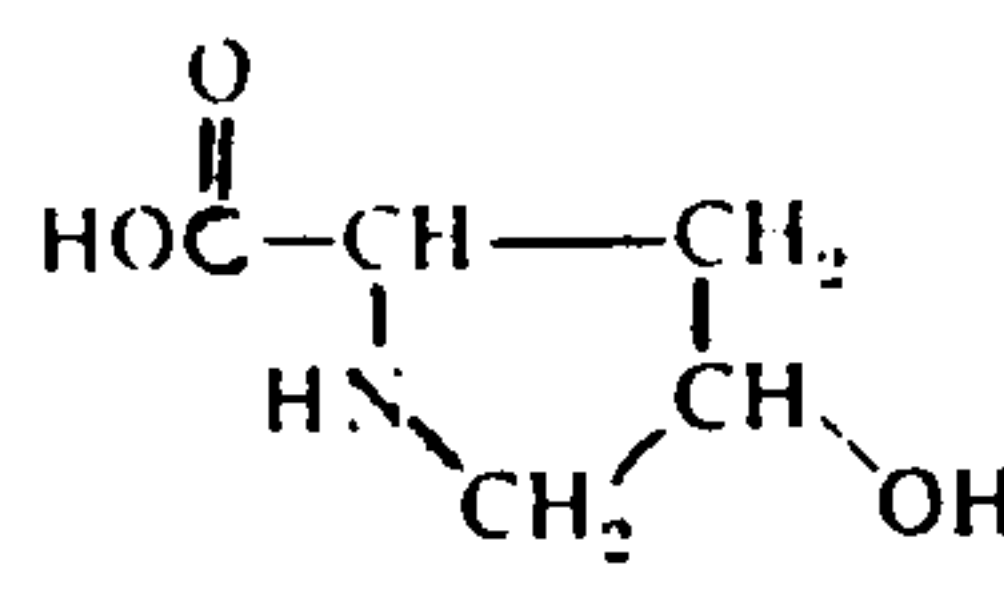


Figure 1.2. Amino acids as dipolar ions: a) the various ionic forms present and b) the titration curve of a typical amino acid. The ionic species predominating at various pH values are shown in boxes.

Table 1.1. The twenty two biologically occurring amino acids.

STRUCTURE OF R	NAME	ABBREVIATION		$pK_{a_1}$ $\alpha\text{-CO}_2\text{H}$	$pK_{a_2}$ $\alpha\text{-NH}_3^+$	$pK_{a_3}$ R Group	$pI$
<b>R group is neutral</b>							
$-\text{H}$	Glycine	Gly	G	2.3	9.6		6.0
$-\text{CH}_3$	Alanine	Ala	A	2.3	9.7		6.0
$-\text{CH}(\text{CH}_3)_2$	Valine <sup>e</sup>	Val	V	2.3	9.6		6.0
$-\text{CH}_2\text{CH}(\text{CH}_3)_2$	Leucine <sup>e</sup>	Leu	L	2.4	9.6		6.0
$-\text{CH}(\text{CH}_2\text{CH}_3)_2$	Isoleucine <sup>e</sup>	Ile	I	2.4	9.7		6.1
$-\text{CH}_2-\text{C}_6\text{H}_5$	Phenylalanine <sup>e</sup>	Phe	F	1.8	9.1		5.5
$-\text{CH}_2\text{CONH}_2$	Asparagine	Asn	N	2.0	8.8		5.4
$-\text{CH}_2\text{CH}_2\text{CONH}_2$	Glutamine	Gln	Q	2.2	9.1		5.7
$-\text{CH}_2-\text{C}_8\text{H}_6\text{N}_2$	Tryptophan <sup>e</sup>	Trp	W	2.4	9.4		5.9
	Proline	Pro	P	2.0	10.6		6.3
(complete structure)							
<b>R contains an <math>-\text{OH}</math> group</b>							
$-\text{CH}_2\text{OH}$	Serine	Ser	S	2.2	9.2		5.7
$-\text{CH}(\text{OH})\text{CH}_3$	Threonine <sup>e</sup>	Thr	T	2.6	10.4		6.5
$-\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$	Tyrosine	Tyr	Y	2.2	9.1	10.1	5.7
	Hydroxyproline	Hyp		1.9	9.7		6.3
(complete structure)							
<b>R contains sulfur</b>							
$-\text{CH}_2\text{SH}$	Cysteine	Cys	C	1.7	10.8	8.3	5.0
$-\text{CH}_2-\text{S}-\text{CH}_2-$	Cystine	Cys-Cys		$\begin{cases} 1.6 \\ 2.3 \end{cases}$	$\begin{cases} 7.9 \\ 9.9 \end{cases}$		5.1
$-\text{CH}_2\text{CH}_2\text{SCH}_3$	Methionine <sup>e</sup>	Met	M	2.3	9.2		5.8
<b>R contains a carboxyl group</b>							
$-\text{CH}_2\text{COOH}$	Aspartic acid	Asp	D	2.1	9.8	3.9	3.0
$-\text{CH}_2\text{CH}_2\text{COOH}$	Glutamic acid	Glu	E	2.2	9.7	4.3	3.2
<b>R contains a basic amino group</b>							
$-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$	Lysine <sup>e</sup>	Lys	K	2.2	9.0	10.5	9.8
$-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}-\text{C}(=\text{NH})-\text{NH}_2$	Arginine	Arg	R	2.2	9.0	12.5	10.8
$-\text{CH}_2-\text{C}_4\text{H}_3\text{N}_2$	Histidine	His	H	1.8	9.2	6.0	7.6
<sup>e</sup> = essential amino acids							

#### 1.4. PRIMARY STRUCTURE AND THE PEPTIDE BOND

The primary structure of a protein is the sequential order of the amino acid residues along the backbone of the protein chain. The amino acids are covalently linked through a peptide bond formed by the formal elimination of  $\text{H}_2\text{O}$  between the OH of the carboxylic acid group of one amino acid and one H of the amino group of a second amino acid (Fig. 1.3).

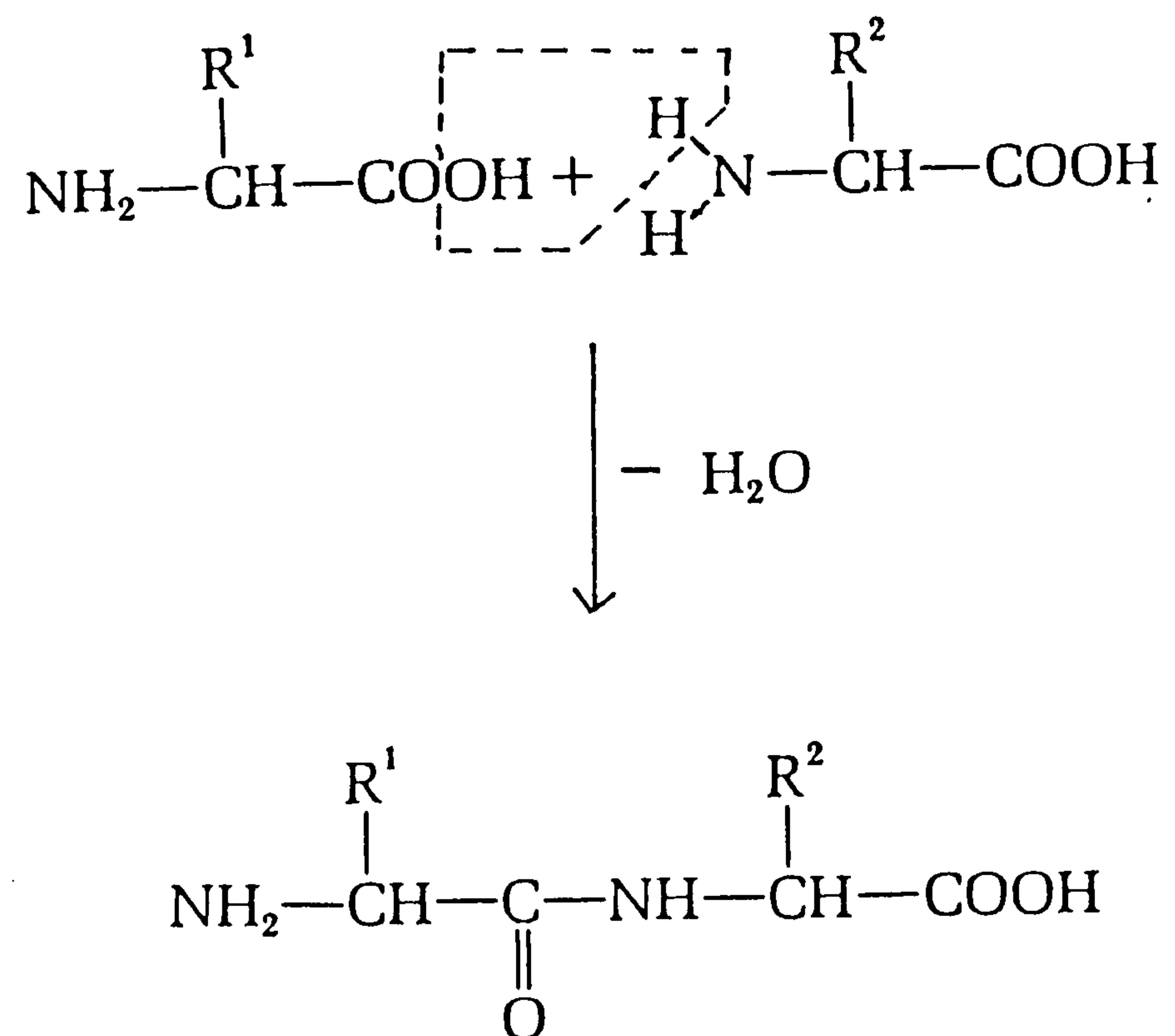


Figure 1.3. The peptide bond, formed by the formal elimination of  $\text{H}_2\text{O}$  between two amino acids.



The protein can thus be viewed as a series of peptide bonds with different sidechains branching out, the nature of the sidechain depending on the amino acid residue. Primary structures of thousands of proteins are known and it is customary to abbreviate them using an alphabetic code for each amino acid residue. The three letter and one letter abbreviations are listed in Table 1.1.

In determining the primary structure, the first thing to learn is the number and size of polypeptide chains in the protein. Automated analytical procedures are then used to establish the amino acid sequence; the stepwise degradation of chains starting at the amino terminus is a widely used procedure.

Many protein chains are crosslinked, either intra- or inter-molecularly; the number and type of crosslinks must be determined for the complete primary structure to be elucidated. The most common form of crosslinkages are disulphide bonds (Fig. 1.4a) formed by oxidation of pairs of cysteines to produce the cystine form mentioned previously. A knowledge of these bonds is also useful in providing information about the secondary and tertiary structures of proteins. Various other kinds of crosslinks have been identified in proteins but do not occur as frequently as cystine. For example, in rigid, fibrous proteins, the chains can be crosslinked when the  $\xi$ -amino group

of lysine is converted to an aldehyde by lysyloxidase; various reaction pathways can then lead to crosslinks involving up to four polypeptide chains (Fig. 1.4b).

Knowledge of the primary structure is only the first step in elucidating the complete structure of a protein, but knowing the primary structure can enable predictions to be made as to the secondary and tertiary structures, since these are determined by favourable interactions between amino acid residues.

## 1.5. SECONDARY STRUCTURE OF PROTEINS

### 1.5.1. Conformations of the Peptide Bond

The illustration of the peptide bond given in Fig. 1.3 is not satisfactory when protein conformations are considered. Fig. 1.3 implies that there is free rotation about the peptide C-N bond; this is not the situation as the groups NH and CO interact to produce partial double bond character in the C-N bond (Fig. 1.5a). A consequence of this resonance stabilization is that the atoms C -C'O-NH-C form a planar group, the peptide unit, which has the dimensions given in Fig. 1.5b (Corey & Pauling, 1953).



Planarity of the peptide unit leads to the possibility of *cis* and *trans* isomers about the peptide bond (Fig. 1.5c). The *cis* isomer is known to occur in cyclic peptides such as diketopiperazine, but in open polypeptide chains the *trans* isomer predominates, being more stable by greater than 2 kcal mol<sup>-1</sup> (La Planch & Rogers, 1967). Increased stability of the *trans* form is accounted for by the closeness of contact between adjacent  $\alpha$  carbon atoms in the *cis* form compared to the *trans* form. A noticeable exception to this occurs in proline and hydroxyproline (Fig. 1.6), where the cyclic nature of the amino acid causes both *cis* and *trans* isomers to have a carbon carrying a hydrogen as third neighbour to the previous  $\alpha$  carbon atom. Consequently, the respective energies of the *cis* and *trans* configurations of imino acids are closer to each other than for the other amino acids in proteins.

The planar peptide units in the protein are linked to each other at the  $\alpha$  carbon atoms. The linking bonds have single bond character, allowing freedom of rotation of the peptide units about the  $\alpha$  carbons (Fig. 1.7). The relative orientation of adjacent peptide units can therefore be adequately described by the dihedral angles,  $\phi$  and  $\psi$ , shown in Fig. 1.7, and consequently the complete backbone conformation of a polypeptide chain can be described knowing the values of  $\phi$  and  $\psi$  for each successive  $\alpha$  carbon, providing the

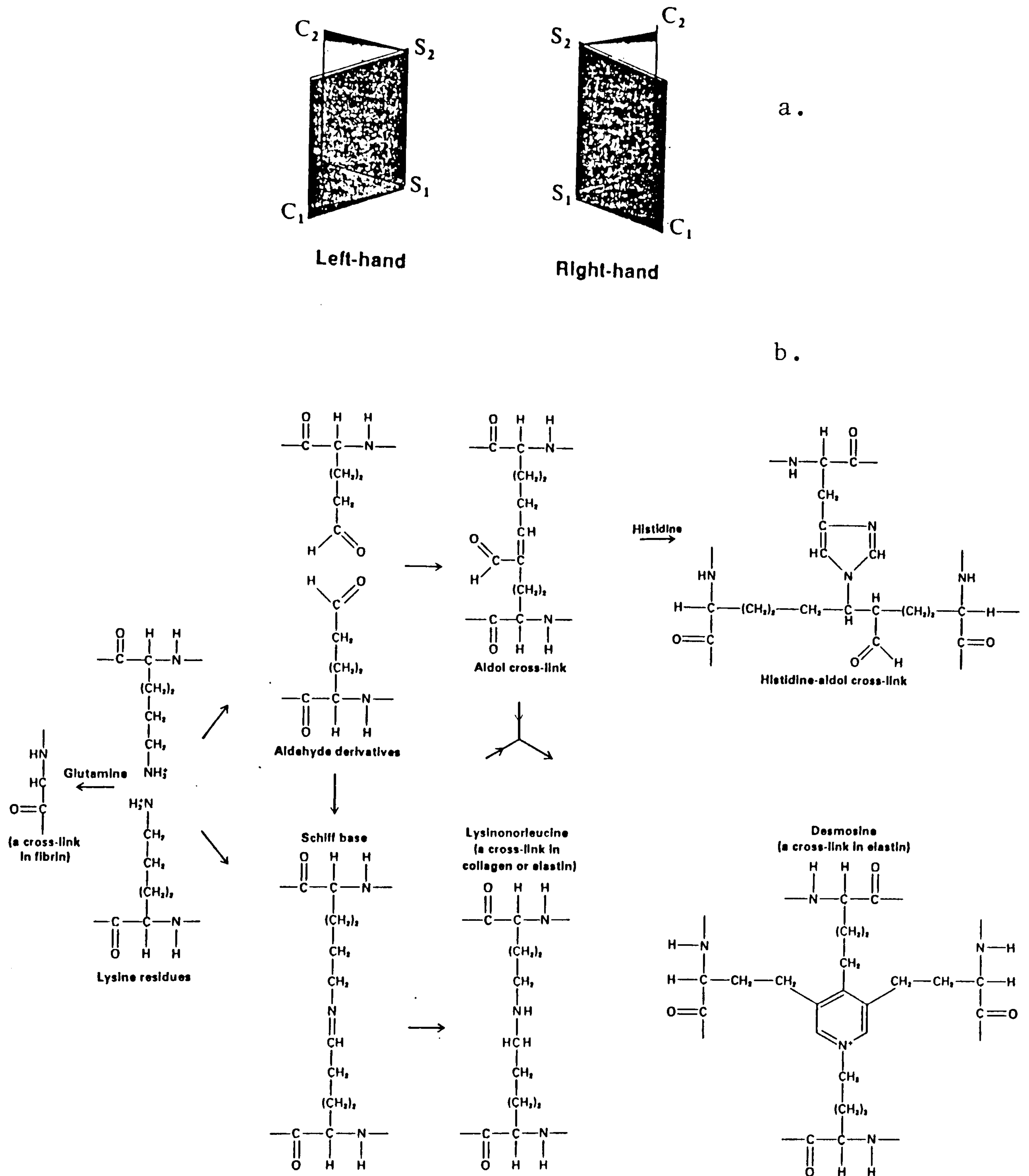


Figure 1.4. The chemical crosslinks formed between protein chains: a) the cystine disulphide crosslink showing two optical isomers and b) the various crosslinks capable of being formed by the oxidation of the  $\epsilon$ -amino group of lysine.



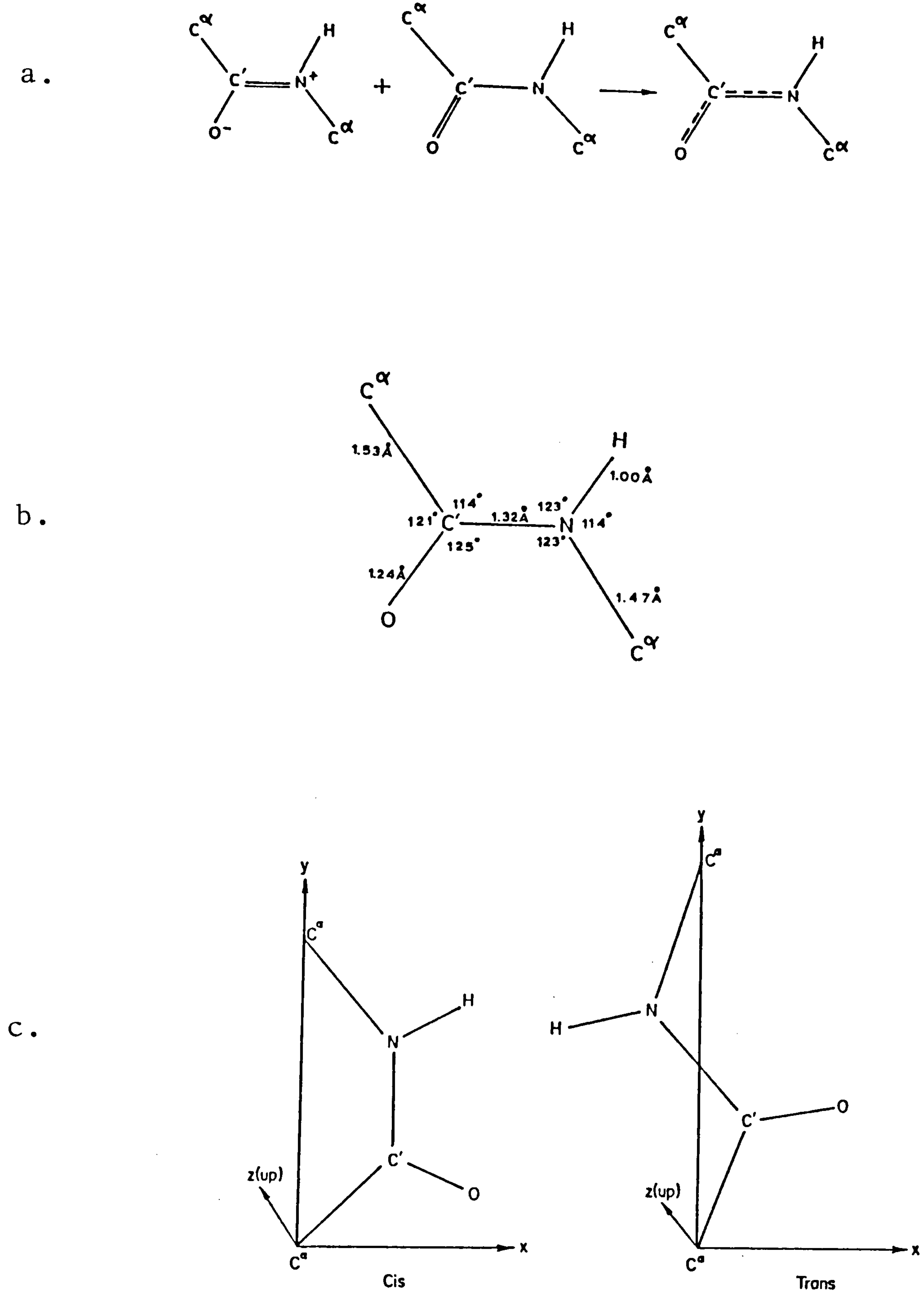


Figure 1.5. a) The two resonant structures of the peptide unit formed by interaction of NH and CO groups producing partial double bond character in the C-N bond.  
 b) The standard dimensions of the peptide unit, according to Corey and Pauling.  
 c) *cis* and *trans* isomeric forms of the peptide unit.

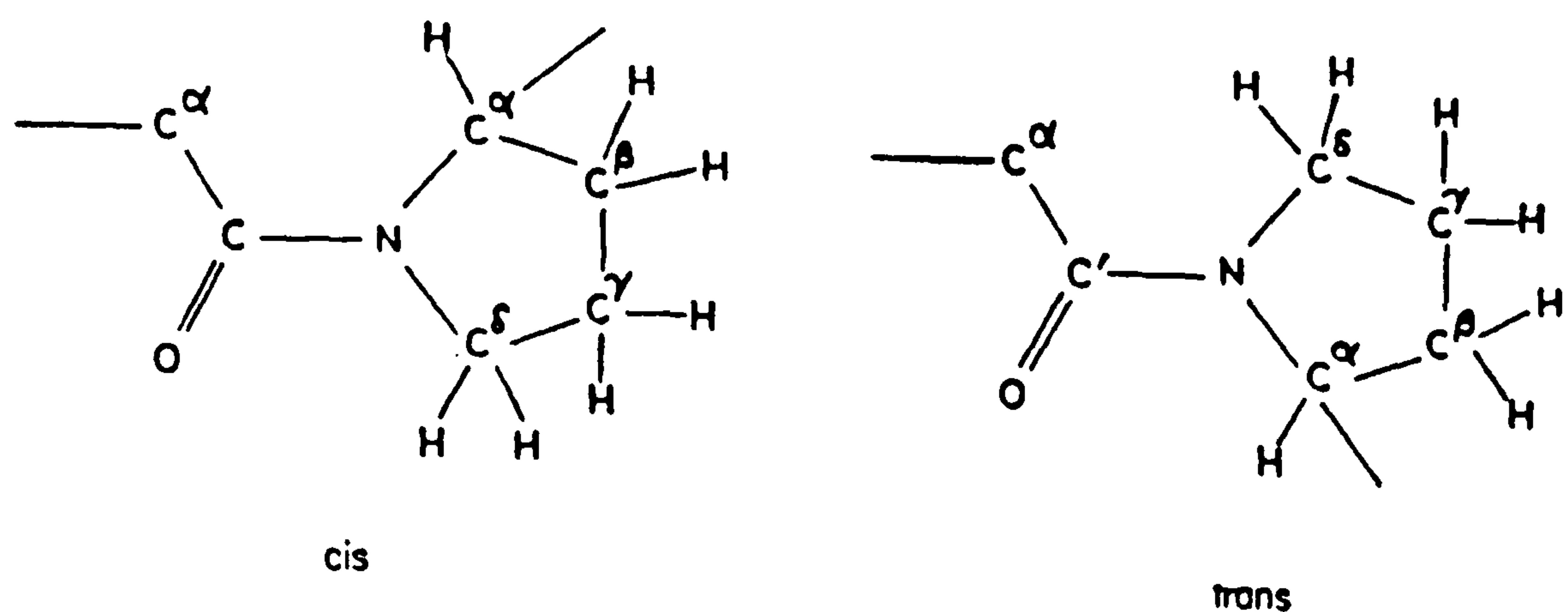


Figure 1.6. *cis* and *trans* peptide units containing a proline side group.

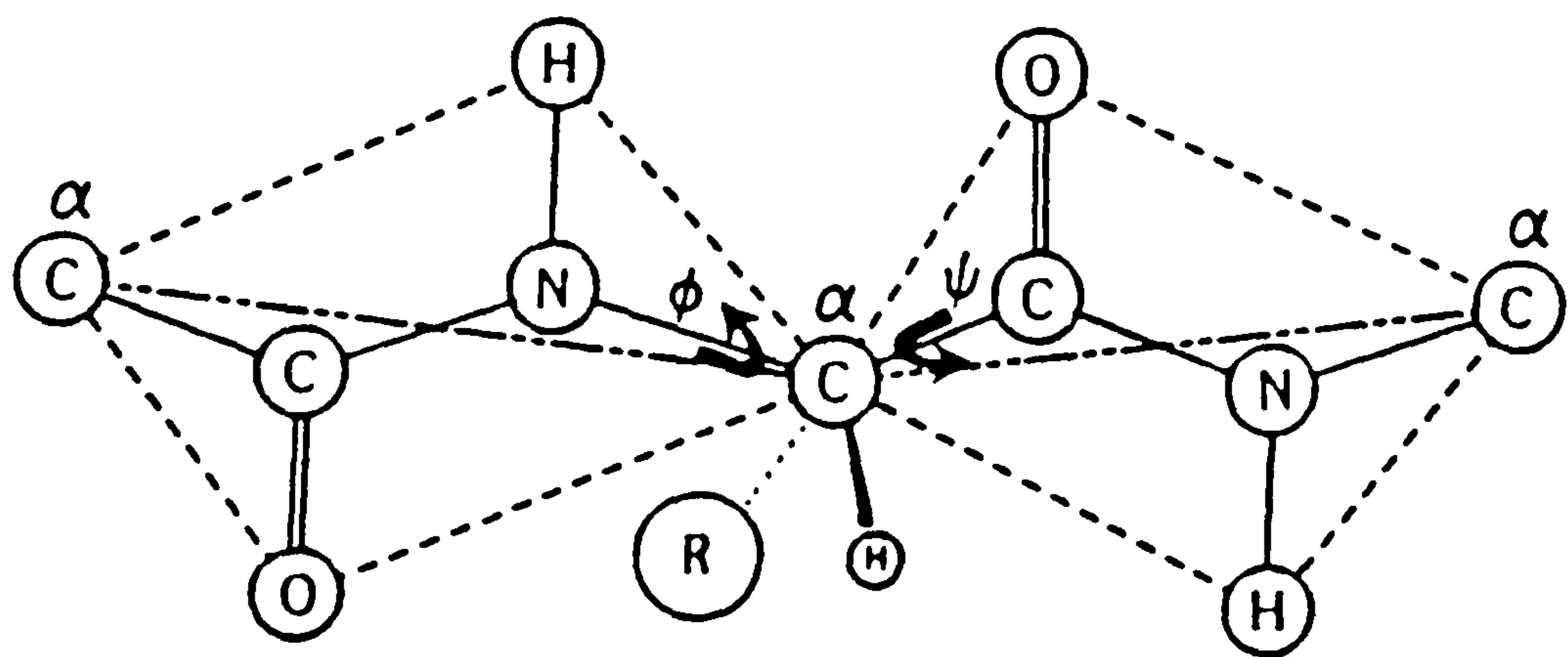


Figure 1.7. Illustration of two peptide units linked through an carbon which allows free rotation of the peptide units. The dihedral angles,  $\phi$  and  $\psi$  are shown.

peptide units are undistorted and all  $\alpha$  carbon bond angles are the same. The last two conditions are not always satisfied and various other parameters are required to define the conformation exactly (see Ramachandran and Sasisekharan 1968).  $\phi$  and  $\psi$  are, however, the most important parameters and given these, a good indication of the peptide chain conformation can be obtained.

### 1.5.2. Helical conformations

#### 1.5.2.1. Energy considerations

For each pair of adjacent peptide units within a polypeptide chain there will be a combination of  $\phi$  and  $\psi$  values corresponding to the conformation of lowest energy. Since the polypeptide will tend towards the most stable structure, these preferred values of  $\phi$  and  $\psi$  would be expected to be repeated throughout the chain, giving rise to a regular, repeating helical structure. For a chain composed of monomeric units a helical conformation can be specified by two parameters; the number of units per turn ( $n$ ) and the projected height of a unit along the helical axis ( $h$ ). The values of  $\phi$  and  $\psi$  for such a structure in a polypeptide chain are necessarily the same at each  $\alpha$  carbon. Relationships between the helical parameters  $n$  and  $h$ , and the conformational parameters  $\phi$  and  $\psi$ , have been determined geometrically for planar peptide units



(Ramachandran and Sasisekharan 1968). Typical helical secondary structures proposed for proteins are shown in Table 1.2 with the corresponding values of  $\phi$  and  $\psi$ .

Theoretically, a chain molecule can adopt an infinite variety of backbone conformations. In proteins, however, steric restrictions limit the values of  $\phi$  and  $\psi$  so that only certain allowed conformations are possible. Allowed values of  $\phi$  and  $\psi$  have been calculated using atomic contact distances, for dipeptide units (Ramachandran and Sasisekharan 1968), and can be represented in a Ramachandran plot showing fully allowed, partially allowed and disallowed regions as a function of  $\phi$  and  $\psi$ , for different amino acid residues in a polypeptide chain (Fig. 1.8a,b).

For glycine ( $R = H$ ) the Ramachandran plot (Fig. 1.8a) is symmetrical, which is a consequence of the achiral atomic structure of glycine. The allowed conformations of alanine ( $R = CH_3$ ) are smaller and assymetric (Fig 1.8b). This is a result of the chirality and unfavourable atomic contacts caused by the  $CH_3$  side chain. Ramachandran plots for residues with longer sidechains, unbranched at the  $\beta$  carbon, are virtually the same as that of alanine. The alanine plot shows three regions of allowed conformation, I, II, and III in which the proposed helical structures of Table 1.2 are located. Region I contains the right-handed  $\alpha, 3_{10}$ ,



TABLE 1.2.

APPROXIMATE VALUES OF DIHEDRAL ANGLES FOR  
SOME REGULAR STRUCTURES

STRUCTURE	$\phi$ (deg)	$\psi$ (deg)
Right handed $\alpha$ helix	123	133
Left handed $\alpha$ helix	237	227
Parallel-chain pleated sheet	61	293
Antiparallel-chain pleated sheet	41	315
Polyglycine II	100	330
Collagen triple helix	135	328

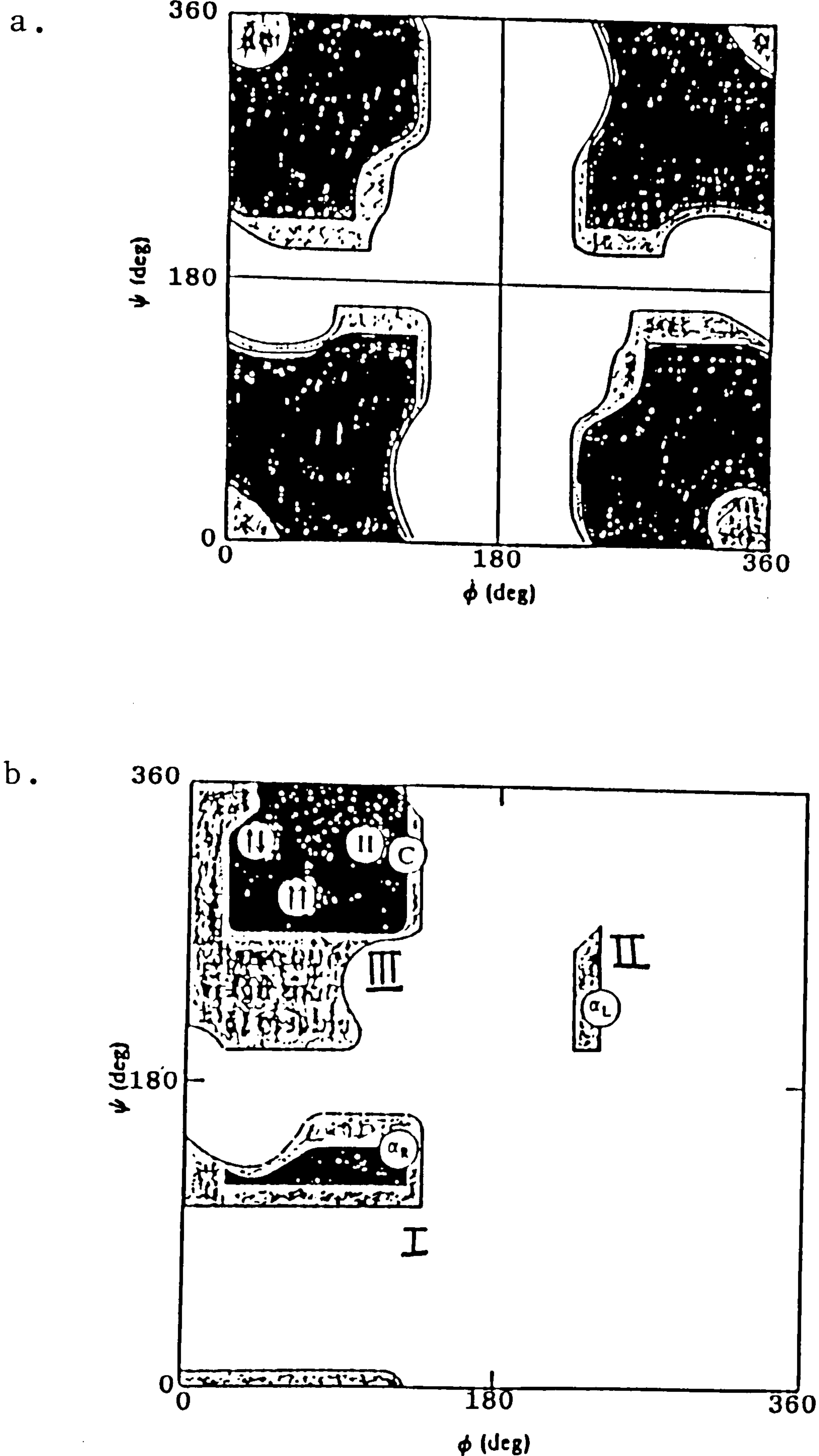


Figure 1.8. Ramachandran plots showing steric contour diagrams for different amino acid residues in a polypeptide chain: a) a glycyl residue and b) an L-alanyl residue. Dark zones show fully allowed regions and light zones partially allowed regions. Also indicated on the alanine plot are the coordinates of the right- and left-handed  $\alpha$  helices ( $\alpha$ ), parallel ( $\uparrow\uparrow$ ) and anti-parallel ( $\uparrow\downarrow$ ) pleated sheets, polyglycine **II** (**II**) and collagen (c) structures. (From Cantor, C.P. and Schimmel, P.R. (1980), *Biophysical Chemistry*, Vol. I, p. 259, W.H. Freeman, San Francisco).

and  $\pi$  helices: region II the left-handed  $\alpha$ ,  $3_{10}$ , and  $\pi$  helices; and region III the  $\beta$  and collagen structures. Region II is only partially allowed and thus the greater stability of right-handed helices is forecast correctly. Extension of the above ideas from a single amino acid in a polypeptide chain to a geometrically-regular helix was made by Venkatachalam and Ramachandran (1967) with resulting Ramachandran plots possessing more restricted areas than obtained for a single amino acid only.

Energy contours indicating the thermodynamically favourable conformations are a further extension of Ramachandran plots so that secondary structures can be considered not only in terms of steric feasibility, but also in terms of overall energy. In calculating the energy contours, three types of interaction are normally included (Bell and Bell, 1988):-

- 1) Non bonded interactions
- 2) Dipolar interactions
- 3) Intrinsic torsional potential

The energies of these interactions can be calculated for specific values of  $\phi$  and  $\psi$ , the total energy of a particular conformation,  $E(\phi, \psi)$ , being the sum of the individual contributions from each interaction.



Energy contours calculated in this way are shown in Fig. 1.9a and 1.9b for a glycine and an alanine residue in a polypeptide chain, respectively. As with the Ramachandran plot (Fig. 1.8a), the energy map for glycine is symmetrical whereas that of alanine has three areas of low energy, corresponding to the allowed regions in Fig. 1.8b.  $\phi$  and  $\psi$  parameters for the proposed right-handed and left-handed helices lie close to the minima of regions I and II, whereas the region of lowest overall energy, region III, corresponds to  $\beta$  structure. If dipolar contributions are omitted from the energy calculations, region I has a lower energy than region III, emphasizing the importance of dipolar interactions in determining protein secondary structure.

Alanine can be considered as representative of most amino acids containing a sidechain, and energy contour maps of most other amino acids have the same characteristic low energy regions. One notable exception to this is proline: because of the rigid pyrrolidine group, the value of  $\phi$  is fixed at approximately  $120^\circ$ , a plot of energy versus  $\psi$  having two minima at  $\psi = 120^\circ$  (region I on the alanine plot) and  $\psi = 325^\circ$  (region III on the alanine plot).  $\psi = 120^\circ$  corresponds to a compact conformation which is often observed in turns or bends in protein chains, whereas the more extended conformation,  $\psi = 325^\circ$ , is characteristic of the values obtained for collagen and



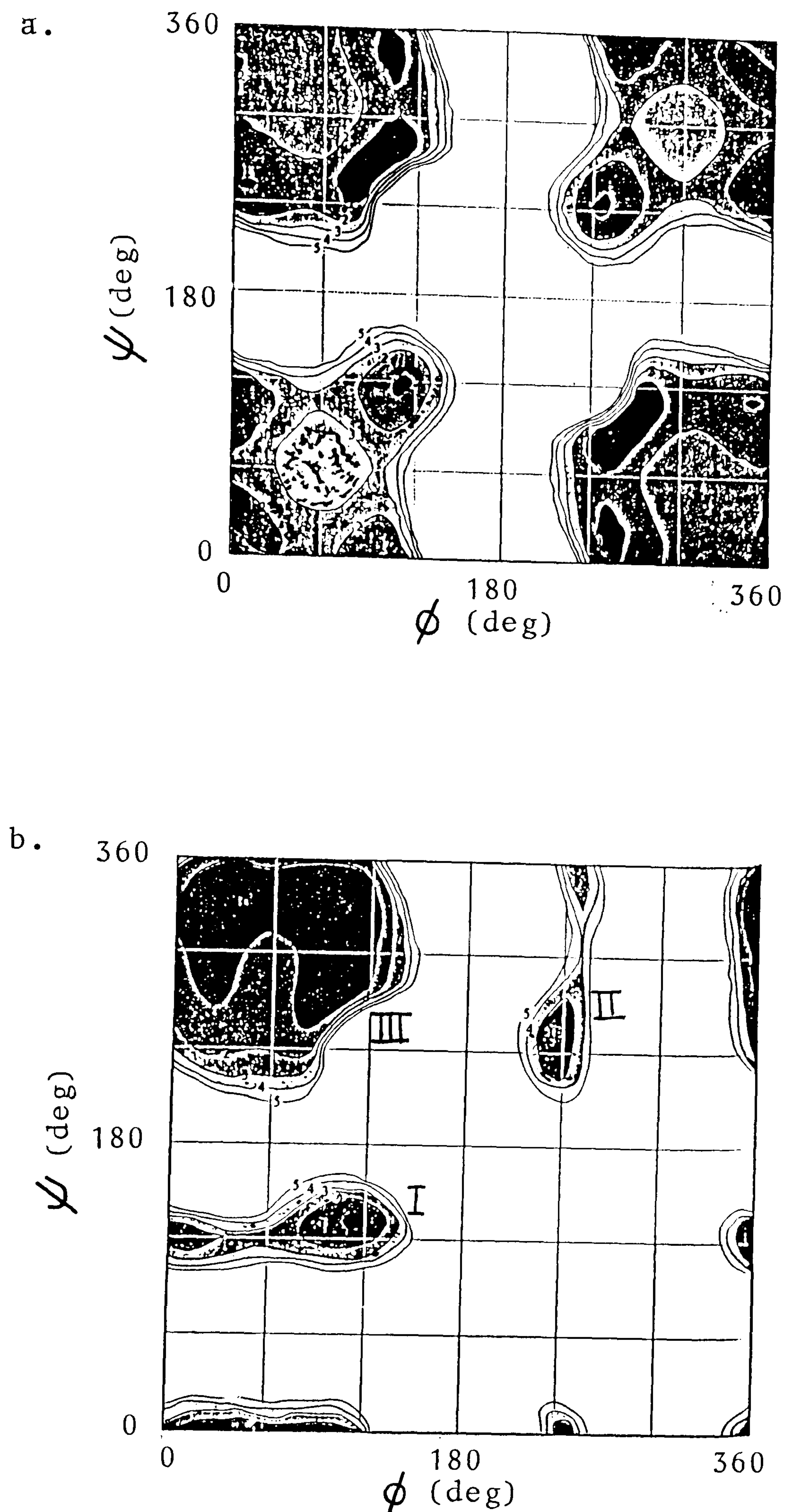


Figure 1.9. Energy contour diagrams for a glycyl residue (a) and an L-alanyl residue (b) in a polypeptide chain. Changes in shading show 1-k cal intervals. (From Cantor, C.P. and Schimmel, P.R. (1980), *Biophysical Chemistry*, Vol. I, p267, W.H. Freeman, San Francisco).

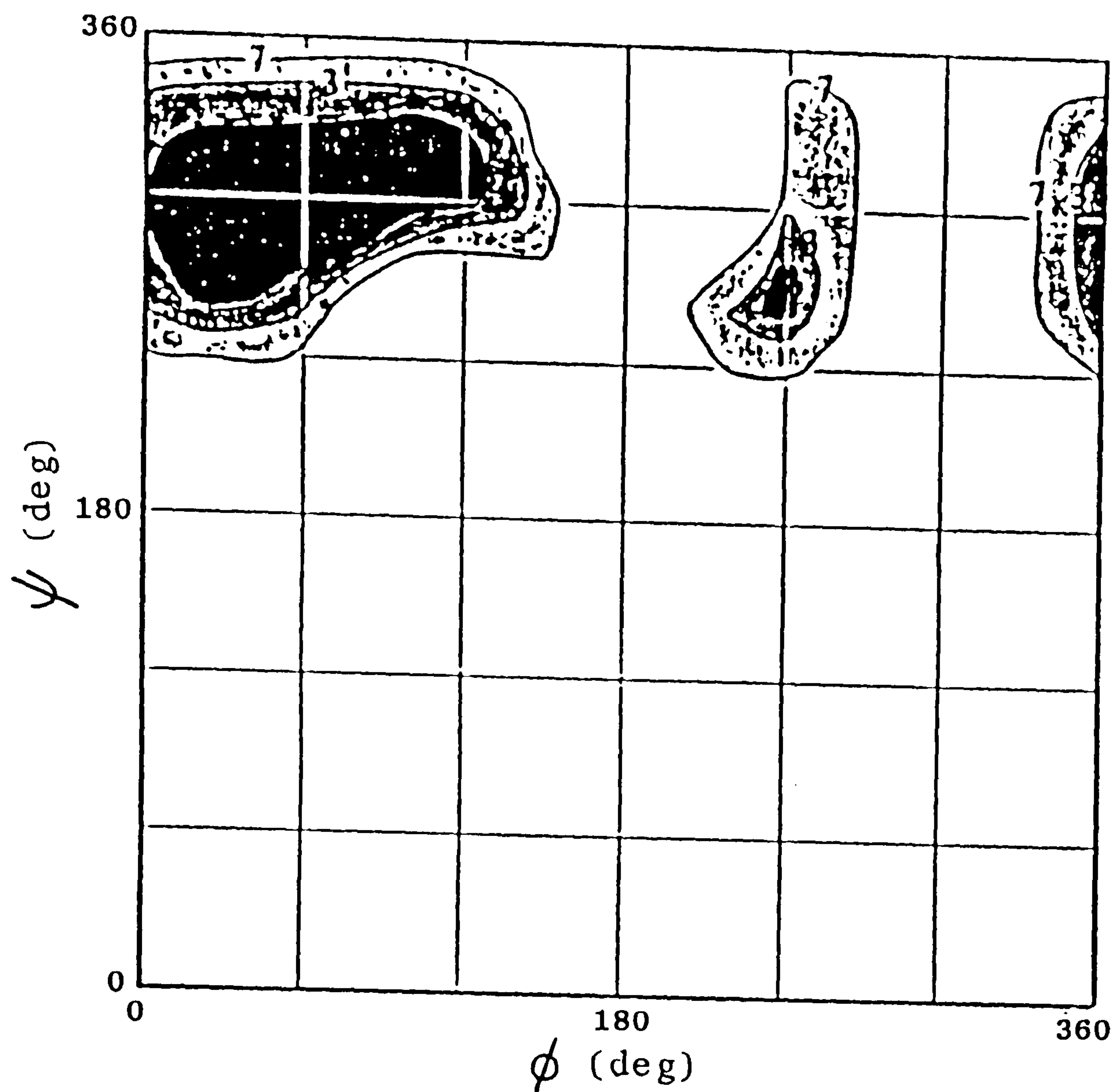


Figure 1.10. Energy contour diagram for an L-alanyl residue that is succeeded by proline. Region 1 of Fig. 1.9b is forbidden; thus a helical structures are not an available conformation. (From Cantor, C.P. and Schimmel, P.R. (1980), *Biophysical Chemistry*, Vol. I, p. 271, W.H. Freeman, San Francisco).

poly-proline structures (see Fig. 1.8b). The most important point concerning proline residues, however, is the effect they have on the energy map of the residue preceeding them. Steric restrictions involving the atoms of the pyrrolidine ring mean that region I of the alanine energy map is forbidden (Fig. 1.10), and consequently the  $\alpha$  helix is not an available conformation. Proline residues are thus never found in  $\alpha$  helical structures unless they are the first residue.

#### 1.5.2.2. Specific helical conformations and their stabilization

The most well-known of protein secondary structures are the  $\alpha$  helix,  $\beta$  sheet and  $\beta$  turn. Generally speaking, if four or more consecutive residues have  $\phi$  and  $\psi$  angles within  $40^\circ$  of  $(120^\circ, 130^\circ)$ , the region of peptide is in a right-handed  $\alpha$  helix. If three or more residues have  $\phi$  and  $\psi$  angles within  $40^\circ$  of  $(60^\circ, 290^\circ)$  or  $(40^\circ, 315^\circ)$ , the structure is  $\beta$  strand (either parallel or antiparallel).  $\beta$  Turns consist of four consecutive residues where the polypeptide chain folds back on itself by approximately  $180^\circ$ , proline often occurring in the second position (Bell and Bell, 1988). These structures have been elucidated through x-ray diffraction of proteins and, particularly, homopolypeptides. Various factors influence the



stability, the most important being hydrogen bonding and the polarity of the amino acid residues.

### $\alpha$ -helix

The right-handed  $\alpha$  helix is illustrated in Fig. 1.11. Its basic features are 3.6 residues per turn with a translation along the helical axis of 1.5Å per residue, giving an overall pitch of 5.4Å and diameter of approximately 6Å; the values of the conformational parameters  $\phi$  and  $\psi$  are 123 and 133 for the "perfect"  $\alpha$  helix. The high stability of the  $\alpha$  helix is attributed to the "intra helix" hydrogen bonding. Neglecting end effects, each peptide carbonyl group is hydrogen bonded to the NH donor four residues along the chain so that, when the helix is viewed with the N-terminus at the top, all the carbonyls point up and the NH groups down (Fig. 1.11). The side chains point away from the helix, a consequence of the L configuration of the amino acids, making it possible for the structure to incorporate almost any sidechain (including those of large hydrophobic residues). As discussed above, however, the rigid pyrrolidine group of proline and hydroxyproline is incapable of packing into such a structure as it prevents the preceeding residue acquiring the conformational angles required for the  $\alpha$  helix and also, because there is no NH group to hydrogen bond. Other residues also influence the



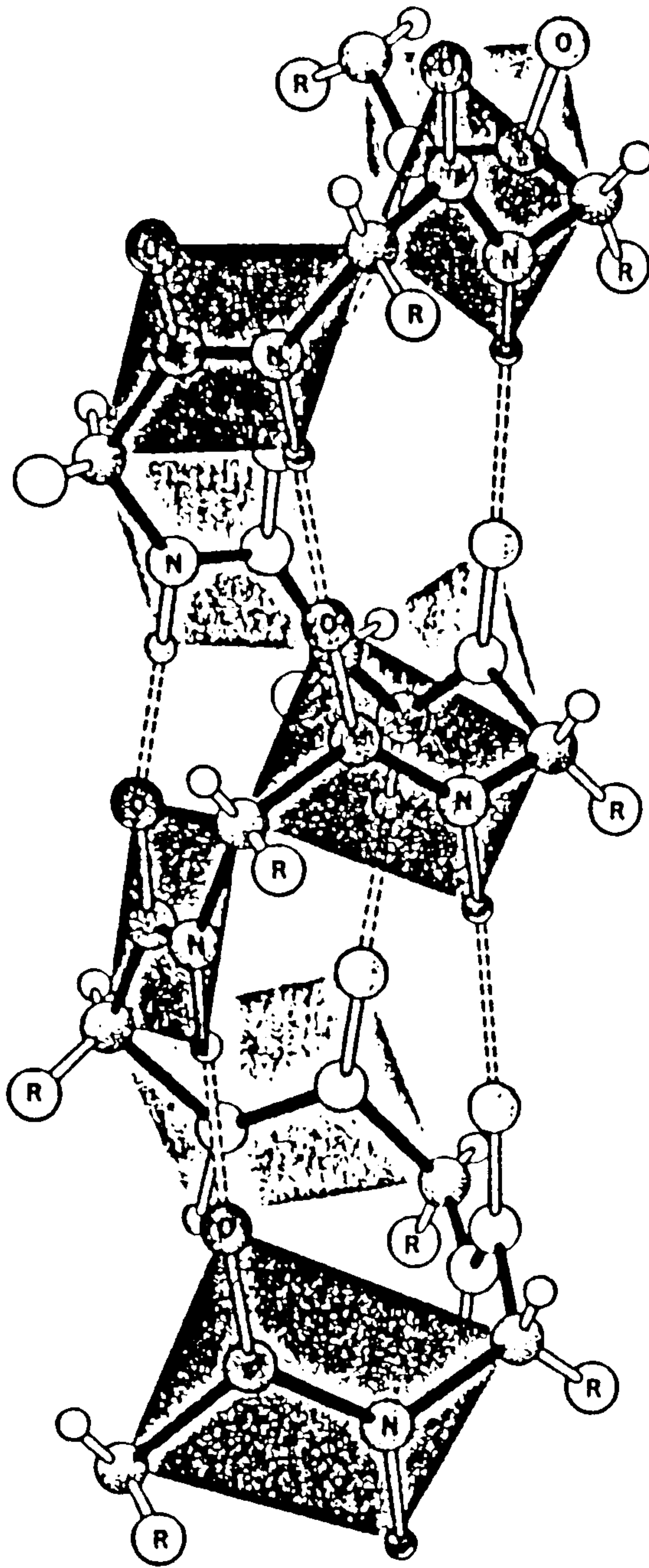


Figure 1.11. Illustration of the right-handed  $\alpha$ -helix. The structure is stabilized by intramolecular hydrogen bonding.

stability of the  $\alpha$  helix, particularly through electrostatic interactions which are critically dependent on pH and ionic strength.

### $\beta$ strand

The  $\beta$  strand structure is an extended conformation of the polypeptide chain, reducing steric hindrance to a minimum. The strand is a two-fold helix, i.e. a pleated structure, with  $\phi$  and  $\psi$  approximately  $50^\circ$  and  $300^\circ$  respectively. The strands associate together via "inter helix" hydrogen bonds (Fig. 1.12) to give sheet structures in which all the peptides participate in hydrogen bonding. The strands forming the sheets may come from quite widely separated regions of the polypeptide chain and can be arranged parallel or antiparallel. Alternate sidechains lie on opposite sides of the sheet structure which is of significance when tertiary packing is considered.

### $\beta$ turn

The  $\beta$  turn or  $\beta$  bend (Fig. 1.13) is a structure which enables the chain to turn through  $180^\circ$  and still maintain energetically favourable conformations. It is stabilised by hydrogen bonds and frequently contains a proline residue in the second or third position in the compact conformation mentioned previously.

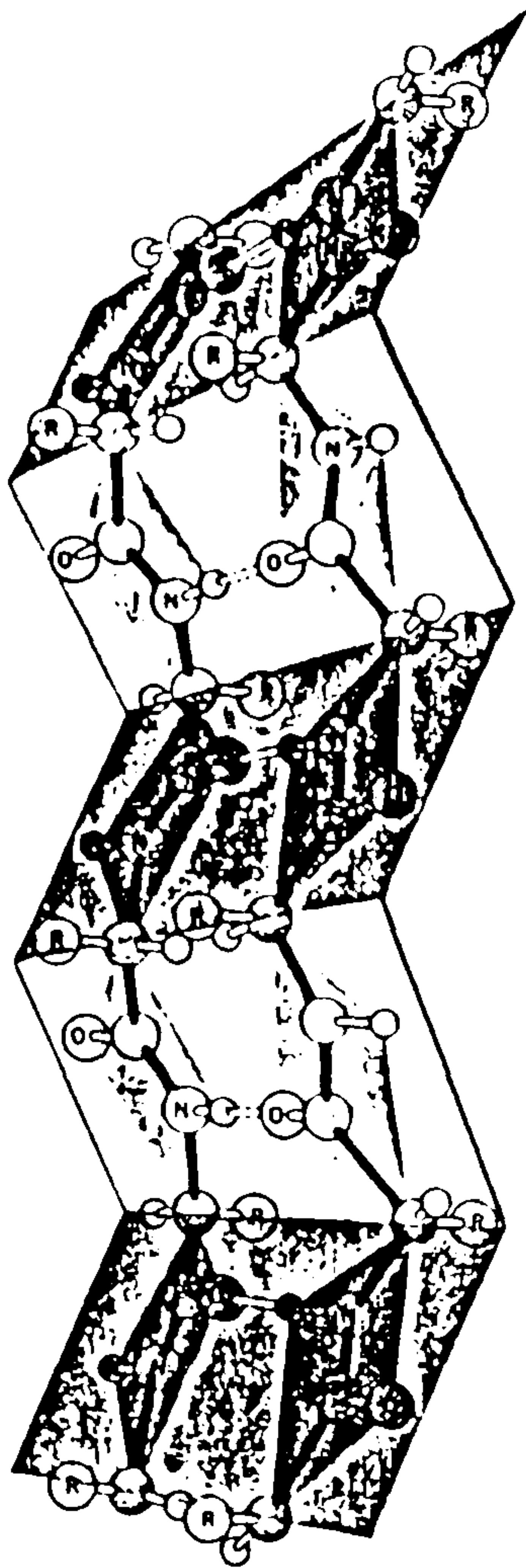


Figure 1.12. The  $\beta$ -sheet structure of proteins showing two anti-parallel  $\beta$ -sheet strands stabilized by interchain hydrogen bonding.

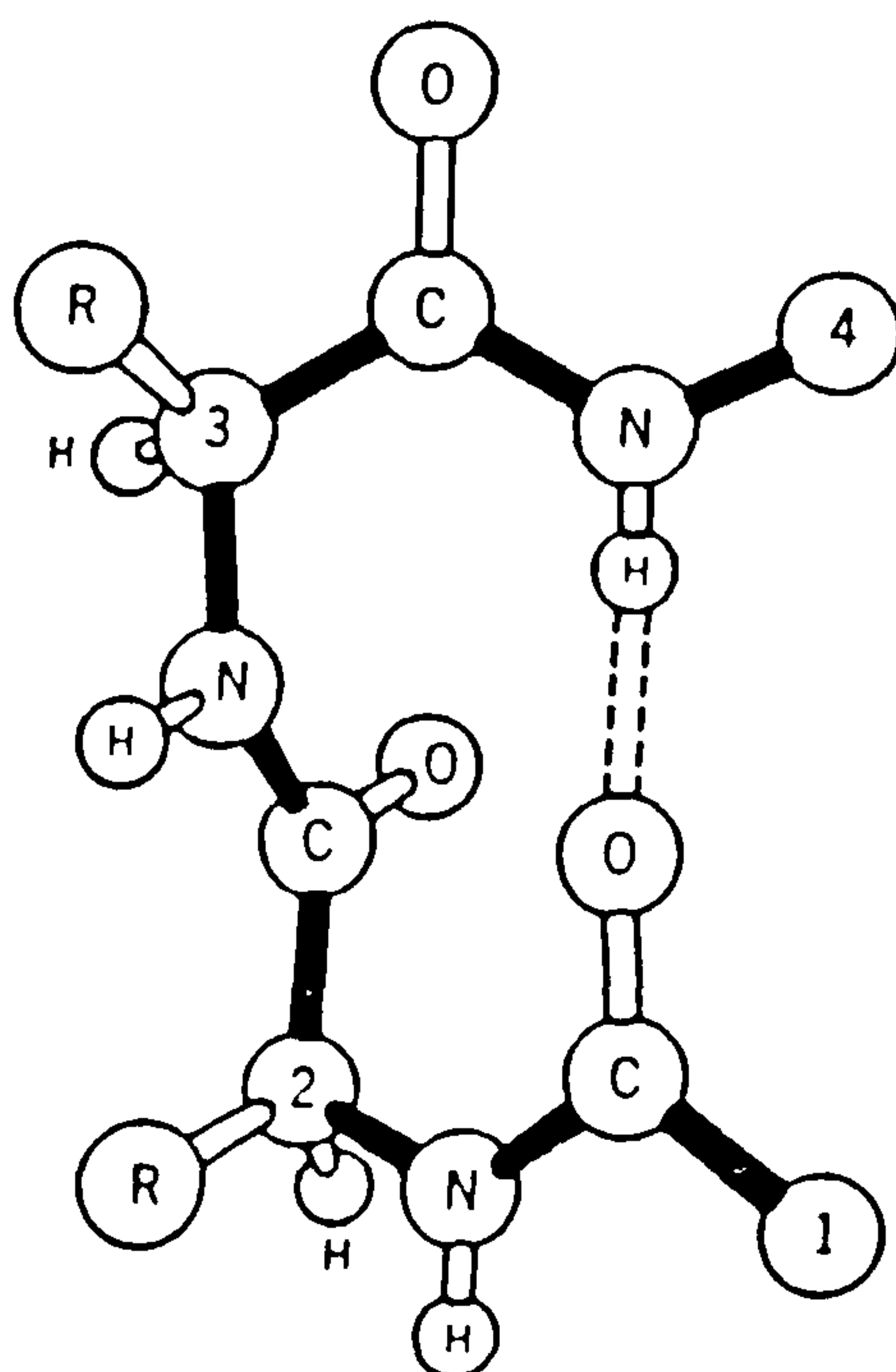


Figure 1.13. The  $\beta$ -turn conformation, allowing the protein chain to turn through  $180^\circ$  and still maintain favourable conformations.



## Polyproline helices and collagen

The final set of common polypeptide secondary structures are the polyproline helices. Poly-L-proline is incapable of forming the  $\alpha$  structure due to steric constraints, but it is able to form two single-stranded helices (polyproline I and polyproline II) that are unique among polypeptide secondary structures in that they contain no hydrogen bonding. Polyproline I is a left-handed helix with 10 residues per 3 turns; all the peptide bonds are in the *cis* form which is extremely rare in peptide structures. Polyproline II is a left-handed helix containing *trans* peptide bonds; the structure has 3 residues per turn and a displacement along the helical axis of 3.12Å per residue. Inter-conversion between the two types can be caused by changes in solvent but under normal aqueous conditions polyproline II is the more stable.

Collagen is unique among proteins in its amino acid sequence, which has approximately 1 in 3 of its residues as proline or hydroxyproline. The high proline content restricts accessible secondary structures to polyproline-like helices and the collagen molecule is composed of three chains, each in the left-handed polyproline II helix, wound into a right-handed superhelix which is stabilised by inter-chain hydrogen bonds. As a consequence of the collagen



triple helical dimensions there is a further primary structure requirement which is that the small glycine residue must occur at every third residue along the polypeptide chain, resulting in triple helical regions always being composed of Gly-X-Y repeating triplets. Collagen structure will be discussed in more detail in Chapter 2.

As can be seen from the above discussion, hydrogen bonds play an extremely important role in directing a polypeptide chain to a particular secondary structure. Other factors such as pH, ionic strength, etc., are also crucial in deciding the type of secondary structure in which a protein may exist. Indeed, as previously mentioned, a simple change of solvent can favour the polyproline I structure over the polyproline II structure. Another example of such a transition is the  $\alpha$ -helix  $\rightarrow$   $\beta$  sheet transition for poly-L-lysine.

The study of such transitions provides further insight into the thermodynamic stability of secondary structures and the factors governing this. Various physical techniques are used to monitor the transitions and of particular importance are chiroptical studies, as different chain conformations interact differently with plane polarised light, rotating it to different extents.

Another important consideration in secondary structure stabilisation is the length of the helical conformation. Consider for example the transition from a random coil to an  $\alpha$  helix. Fig. 1.14 is a schematic diagram of the hydrogen bond network. As the hydrogen bonds are formed between the  $(i)$ th and  $(i+4)$ th residue, six dihedral rotation angles are spanned. The formation of the first hydrogen bond involves the loss of a substantial amount of conformational entropy (six dihedral angles becoming fixed) in return for the gain in energy associated with the formation of only one hydrogen bond. All successive residues joining the helix, however, will involve the fixing of only two dihedral angles, a considerably lower entropic barrier, in return for the gain in energy associated with one hydrogen bond. The tendency is thus to form long stretches of helical structure to overcome the initial loss of entropy. This effect also explains the co-operative nature of secondary structure formation.

Finally, the type of secondary structure is dependent on interactions of the amino acid residues with the solvent and also with adjacent residues. Different amino acids occur preferentially in different structures, and from the large data base of known protein structures it is possible to assess the probability of specific amino acids occurring in each type of secondary structure (Table 1.3) in proteins of unknown conformation.

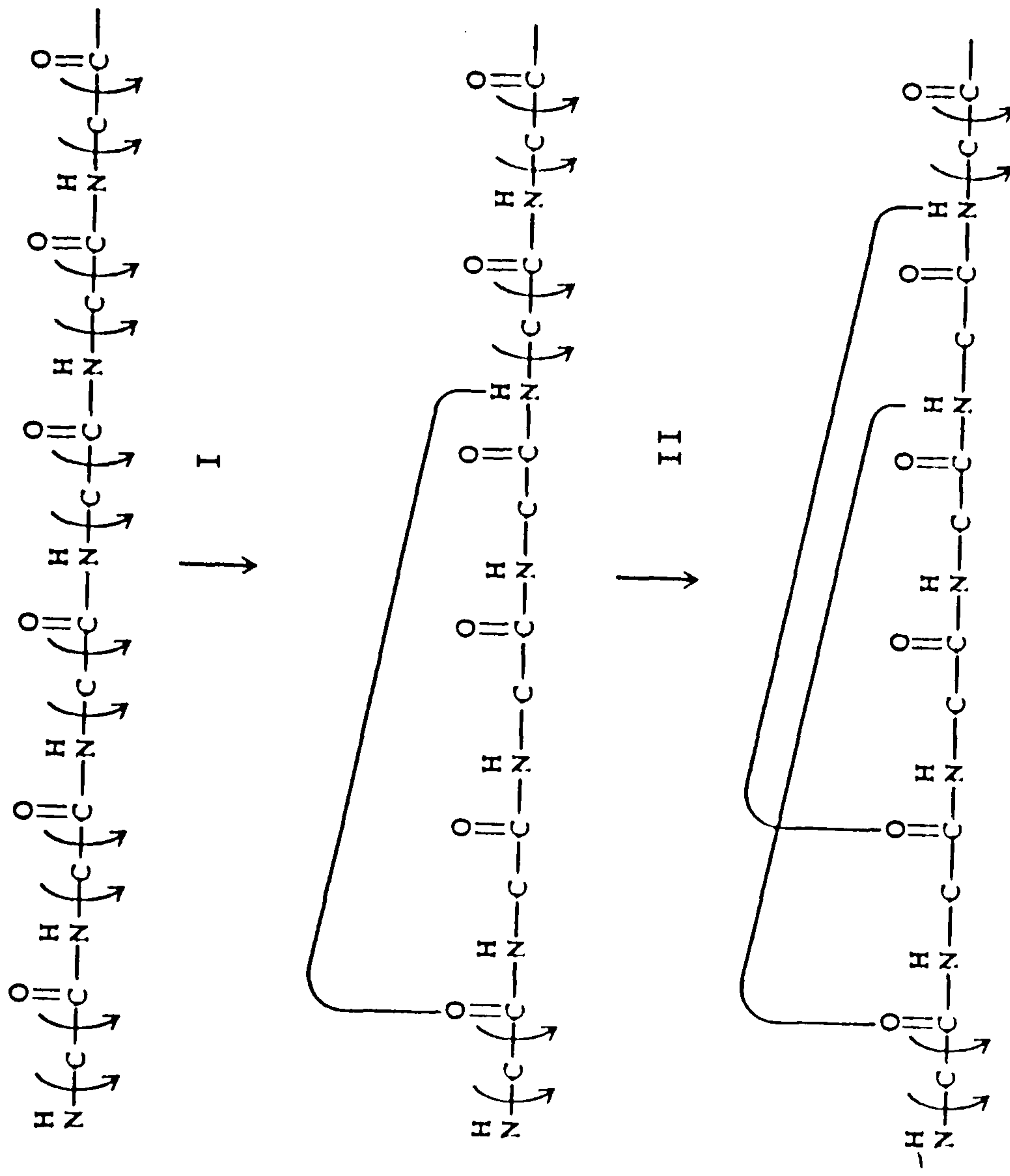


Figure 1.14. Schematic illustration of the six dihedral angles which must become fixed in the initiation of an a helical structure (I) compared with subsequent growth of the helix, involving the fixing of only two dihedral angles (II).



Table 1.3. Classification of amino acid residues as formers breakers or indifferent for  $\alpha$ -helical and  $\beta$ -sheet regions.

Helical residues	Classification	$\beta$ -Sheet residues	Classification
Glu	H <sub><math>\alpha</math></sub>	Met	H <sub><math>\beta</math></sub>
Ala	H <sub><math>\alpha</math></sub>	Val	H <sub><math>\beta</math></sub>
Leu	H <sub><math>\alpha</math></sub>	Ile	H <sub><math>\beta</math></sub>
His	h <sub><math>\alpha</math></sub>	Cys	h <sub><math>\beta</math></sub>
Met	h <sub><math>\alpha</math></sub>	Tyr	h <sub><math>\beta</math></sub>
Gln	h <sub><math>\alpha</math></sub>	Phe	h <sub><math>\beta</math></sub>
Trp	h <sub><math>\alpha</math></sub>	Gln	h <sub><math>\beta</math></sub>
Val	h <sub><math>\alpha</math></sub>	Leu	h <sub><math>\beta</math></sub>
Phe	h <sub><math>\alpha</math></sub>	Thr	h <sub><math>\beta</math></sub>
Lys	I <sub><math>\alpha</math></sub>	Trp	h <sub><math>\beta</math></sub>
Ile	I <sub><math>\alpha</math></sub>	Ala	I <sub><math>\beta</math></sub>
Asp	i <sub><math>\alpha</math></sub>	Arg	i <sub><math>\beta</math></sub>
Thr	i <sub><math>\alpha</math></sub>	Gly	i <sub><math>\beta</math></sub>
Ser	i <sub><math>\alpha</math></sub>	Asp	i <sub><math>\beta</math></sub>
Arg	i <sub><math>\alpha</math></sub>	Lys	b <sub><math>\beta</math></sub>
Cys	i <sub><math>\alpha</math></sub>	Ser	b <sub><math>\beta</math></sub>
Asn	b <sub><math>\alpha</math></sub>	His	b <sub><math>\beta</math></sub>
Tyr	b <sub><math>\alpha</math></sub>	Asn	b <sub><math>\beta</math></sub>
Pro	B <sub><math>\alpha</math></sub>	Pro	b <sub><math>\beta</math></sub>
Gly	B <sub><math>\alpha</math></sub>	Glu	B <sub><math>\beta</math></sub>

NOTE: H = strong former; h = former; I = weak former; i = indifferent; b = breaker; B = strong breaker.

SOURCE: P. Y. Chou and G. D. Fasman, *Biochemistry* 13:222 (1974).

## 1.6. TERTIARY AND QUATERNARY STRUCTURES

### 1.6.1. Generalisations Describing Tertiary Structures

The tertiary structure of a protein is the way in which the secondary structural elements pack together to form the globular molecular units of proteins. A number of general rules have been formulated to describe tertiary structure:-

1. The conformation of individual peptide groups fall into the stable ranges predicted from energy calculation so that the resultant secondary and tertiary structures are limited by the constraints imposed by the allowed dihedral angles.

2. Almost all the polar sidechains in the protein are at the surface of the molecule where they can be solvated, thus stabilizing the molecule. Exceptions to this rule indicate that the 'non-surface' polar groups may be involved in some specific internal function.

3. Non-polar hydrophobic residues are located preferentially in the interior of the molecule in water-soluble globular proteins. Exceptions to this rule are glycine and alanine because of their size, and probably lipid-interacting (e.g. transmembrane) proteins where the hydrophobic residues might be

expected to be on the outside.

4. Virtually all hydrogen bond donors and acceptors are located where they can be satisfied. At the surface the groups tend to be solvated, while in the interior, they are incorporated into ordered secondary structures.

These basic rules were derived from X-ray crystallography of myoglobin by Kendrew *et al.* (1961, 1963), but in general are found to be satisfied for most known protein structures.

#### 1.6.2. Tertiary Structure Determination and Prediction

There are a variety of methods used for protein tertiary structure determination but the results rarely give precise structural information, except in the case of X-ray crystallography, which is the most powerful tool, giving information about the actual positions of the residues in the protein unit. When X-ray studies are not possible, other techniques utilized include:-



1) Solvent perturbation, where small molecules can alter the environment of surface chromophores giving an indication of the solvent exposure of such chromophores.

2) Limited proteolysis in the presence of different ligands: altered susceptibility to proteases is indicative of conformation change.

3) Chemical modification of certain amino acid sidechains indicates the availability of these residues to water-soluble reagents and hence their position within the globular protein (surface or interior).

4) Sedimentation, gel filtration or light scattering give a general picture of the dimensions of the protein unit.

More information however can be obtained by the use of these techniques in conjunction with predictions from the generalisations about tertiary structure described earlier, particularly if the amino acid sequence is known. In a protein a particular  $\alpha$  helix or  $\beta$  sheet will be orientated in the tertiary structure so that it obeys the simple rules. In  $\beta$  sheet structures alternate residues lie on either side of the sheet; if all the residues are hydrophobic the secondary structural element would be expected to be found in

the interior of the molecule. If however, one side of the sheet is hydrophobic and the other is hydrophilic, it would be predicted that the structure would be at the surface of the molecule with the hydrophilic side facing out and the hydrophobic side in. Similarly, a helical regions can be assessed in terms of areas of hydrophobicity by means of 'Edmundson Wheels' (Fig. 1.15). These represent a projection of the helical structure on to a flat surface looking down the helical axis, again showing that the positions of the residues in the primary structure can lead to hydrophobic and hydrophilic sides.

From analysis of  $\beta$  sheet regions of proteins with known crystal structures, two further generalisations have become apparent:-

1) In antiparallel sheet structures branched side chains are next to unbranched side chains to give better packing.

2) In parallel sheet structures branched sidechains are found next to each other as are unbranched.

Obviously, knowing the primary sequence it is now possible to predict whether the  $\beta$  sheet structure will be parallel or antiparallel.

It is therefore possible to obtain a great deal of information regarding tertiary structure if the simple rules are used in conjunction with the experimental techniques described above.

### 1.6.3. Protein Folding and Unfolding

The folding of a protein into the specific three-dimensional tertiary structure and loss of this structure (denaturation) has been the subject of study for many years. The arguments fall into two categories, thermodynamic control and kinetic control.

Studies of proteins such as **staphyloccal nuclease** have shown that complete activity can be recovered after denaturation, supporting the thermo-dynamically controlled mechanism. Temperature jump studies have been used to follow transitions associated with the unfolding of chymotrypsin and ribonuclease and have given single relaxation times consistent with a simple two state model. This again suggests thermodynamic control.

In other proteins such as muscle aldolase, renaturation has lead only to partial recovery of activity, the rate of regain being dependent on the presence of a substrate or other ligand. This suggests that the renaturation process is pathway-dependent



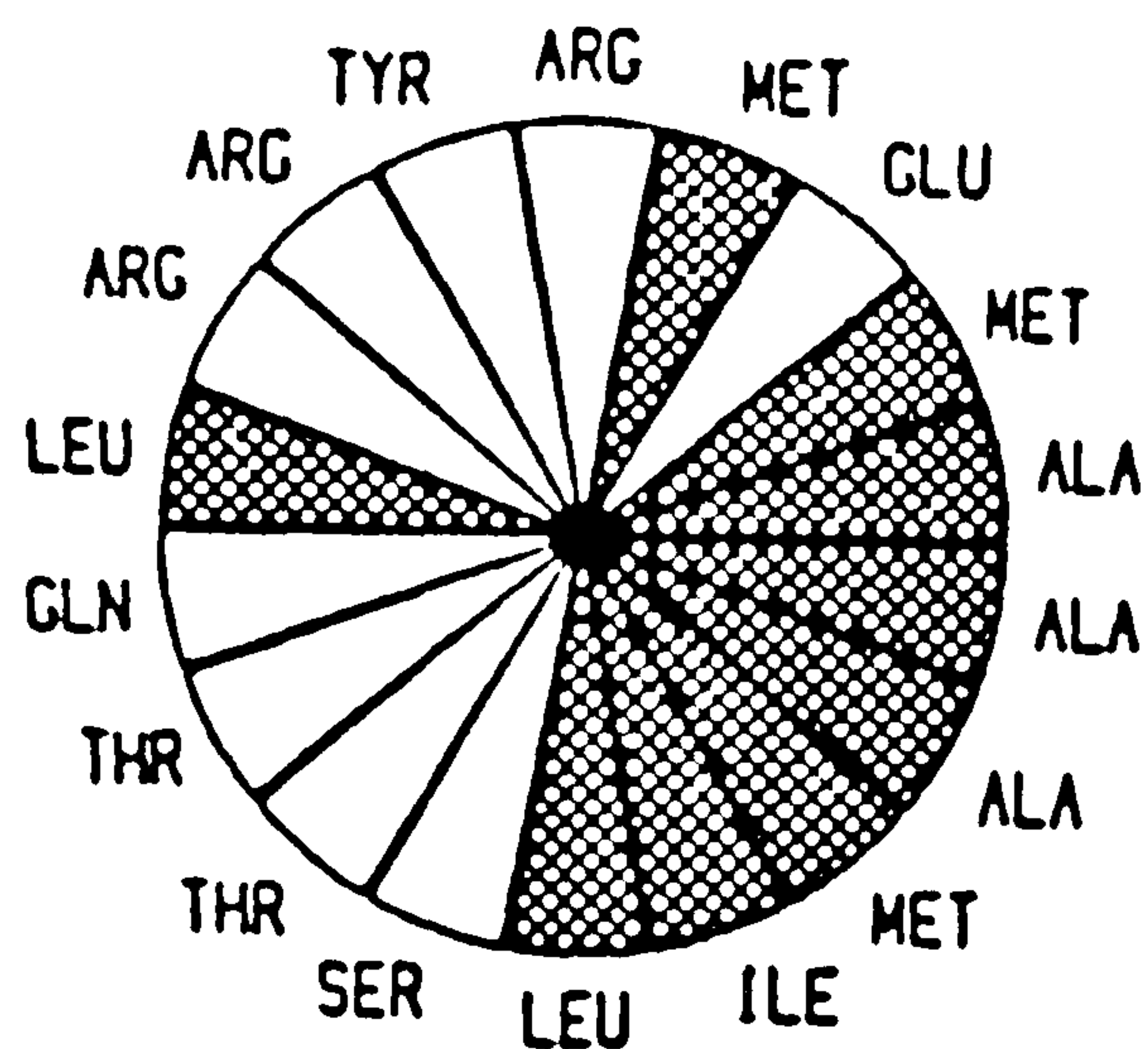


Figure 1.15. Example of an Edmundson wheel illustrating the manner in which helical sequences can have hydrophobic (shaded segments) and hydrophilic (open segments) sides.

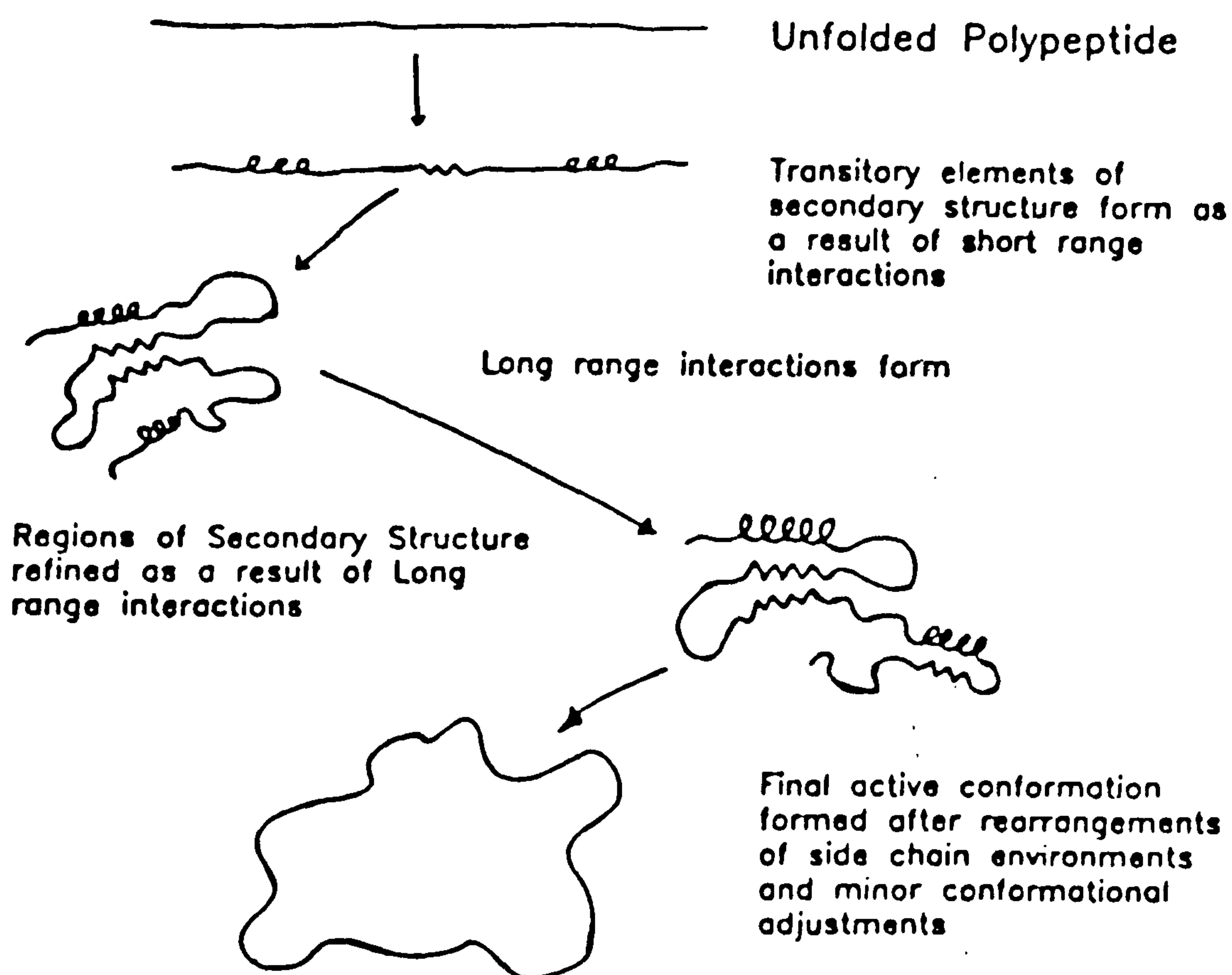


Figure 1.16. Diagrammatic illustration of the way a polypeptide chain may fold into the final native structure through nucleation of areas of local secondary structure which interact favourably with each other.

(i.e. kinetically controlled). There is also evidence that protein folding can occur before complete synthesis of the protein, meaning the complete protein chain is not a prerequisite for folding, again supporting kinetic control.

Most proteins capable of successful, complete renaturation are usually globular proteins of small size while large, often multi-subunit, proteins are incapable of complete renaturation. The folding process cannot be completely random as the time required to attain the correct tertiary structure by random sampling of all possible conformations would be far greater than observed folding times. It therefore seems likely that folding is kinetically controlled, with nucleation events directing the process. The scheme illustrated in Fig. 1.16 indicates the way in which a polypeptide chain may acquire its native structure through nucleation, in different parts of the chain, of areas of local secondary structure which can either interact favourably or unfavourably with one another, directing the folding process. For small proteins the process is rapid, occurring in a co-operative manner, giving the two state kinetics often observed, whereas with larger proteins, there are more possibilities of metastable structures forming, leading to more complex kinetics.

#### 1.6.4. Quaternary Structure

The term "quaternary structure" is used to describe a macromolecular system consisting of non-covalently linked subunits, the assemblies of which are described by the following designations:-

**Stoichiometry** - The number and type of each kind of subunit involved.

**Architecture** - A description of the geometric arrangements of the subunits including the type of symmetry formed.

**Assembly** - A description of subunit interactions within quaternary structure and the nature of interface contacts between subunits.

**Inter-subunit communications** - A description of the ways that conformational changes within one subunit of an oligomer affect the conformations of other subunits within the same oligomer.

The first thing to learn about the quaternary structure is the number and type of subunits. These can range from the simplest possible case of a protein with only two identical subunits, e.g. liver alcohol dehydrogenase, to more complex proteins with multiple numbers of each of several types of subunits, e.g.



pyruvate dehydrogenase [ $t_{24} (P_2)_{12} (F_2)_{12}$ ].

It is also possible to obtain polymeric structures, where the relative composition is fixed but the overall size is not, e.g. microtubules  $[(\alpha\beta)_n]$ .

A variety of techniques are used to learn about the stoichiometry of oligomeric proteins, which usually involve determination of the molecular weight of the oligomeric form before denaturation, and of the separate subunits after denaturation.

An oligomer consisting of a number of subunits could have a large number of spatial arrangements, the number increasing with the number of subunits in the oligomer. The number of possible arrangements are limited, however, by two assumptions which appear to hold in most cases:-

1) All subunits are in equivalent, or pseudo equivalent, environments. Strictly, this applies only to homo-oligomers, but frequently hetero-oligomers can be considered as being composed of multiples of "effective subunits" (e.g.  $\alpha\beta$ ) each containing two subunits.

2) The bonding potentials of the subunits must be saturated.

The assembly of the subunits into the **quaternary** structure can occur in various ways. In homo-oligomers the process can involve stepwise addition of subunits to the growing "core" until the stable oligomeric structure is formed, or stable intermediates may be formed which then associate to give the final structure. With hetero-oligomers the latter seems the most likely. The study of such associations is frequently monitored using techniques such as sedimentation, gel filtration or light scattering.

The **quaternary** structure is determined by the tertiary structural subunits which in turn are determined by the secondary structural elements. It should be noted, however, that the formation of a particular **quaternary** structure from its constituent subunits may, to some extent, affect the tertiary structure and hence possibly the secondary structure of the subunit.

## CHAPTER 2: COLLAGEN & GELATIN

### 2.1. INTRODUCTION

Gelatins are proteinaceous materials which have no existence in nature but are derived from the parent protein collagen by various methods of extraction, which involve the destruction of the collagen secondary structure and, in most cases, parts of the primary structure. Scientific interest in gelatins arose because of their physical properties, which are of great importance in the food and photographic industries. The study of gelatins has also enabled advances to be made in the understanding of the biologically significant parent protein, collagen (Veis, 1964).

Collagen is the major protein component of a wide range of species, occurring in such tissues as bone, skin, tendon, cartilage, cornea and basement membranes. In skin, tendon and cartilage, collagen constitutes over 70% of the dry weight (Grant & Jackson, 1976).

The major function of collagen in the body is as a structural macromolecule. All tissues whose primary function are either weight-bearing (bone, cartilage), transmission of forces (tendons, ligaments),



protection or compartmentalisation (dermis, fascia), transmission of light (cornea, vitreous), or distribution of fluids (blood vessels, glandular ducts) contain collagen as a major component (Bornstein & Traub, 1979). *In vivo* collagen occurs as white, opaque, non-branching fibrils embedded in a matrix of mucopolysaccharide and other protein. The architecture of the fibrils in a particular tissue is determined in part by the genetic collagen type (see Table 2.1) and by the association of collagen or collagens with other structural macromolecules such as proteoglycans and acidic glycoproteins. The diversity in the architecture of the fibres in collagen-containing tissues can be illustrated by considering skin and tendon. In skin, the collagen fibres are in an irregular "loose-weave" allowing for extension in all directions within the plane of the tissue, whereas in tendon, fibre bundles are large and arranged in parallel, giving tensile strengths as great as 20-30 kg/mm<sup>2</sup> of tissue (Bornstein & Traub, 1979).

Regardless of the species and tissues from which the collagen fibres originate, they have many things in common.

- 1) The amino acid content of the protein chains is approximately one-third glycine with the imino acids, proline and hydroxyproline, contributing between

15-30% of the remainder of the chains.

2) A characteristic X-ray diffraction pattern arising from the triple-helix structure (see later).

3) A characteristic banded structure when viewed in the electron microscope (Balian & Bowes, 1977).

The development of ideas with respect to collagen and gelatin throughout the years is reflected in the general nomenclature now used to describe these biopolymer systems. It is useful at this moment to give a general description of the nomenclature to be used throughout this thesis which is the same as that suggested by Bornstein and Traub (1979).

The general term "collagen" has been used to refer to the collagenous component of a tissue or structure, and this is a very loose definition. More specifically, collagen refers to the collagen molecule or monomer which consists of three  $\alpha$  chains and is the basic monomer unit serving as a building block for the collagen fibre. Collagen fibres may have a diameter of 0.1-15  $\mu\text{m}$  and consist of bundles of fibrils, which may range in diameter from about fifty to several thousand Angstroms and are seen in the electron microscope as distinct entities. Fibrils may be composed of small assemblies called microfibrils.



The collagen molecules commonly found in skin, tendon, ligament and bone (type I collagen) consist of two different  $\alpha$  chains,  $\alpha_1$  and  $\alpha_2$ , the molecule being designated by the formula  $[\alpha_1(I)]_2 \alpha_2(I)$ .

Different types of collagen molecules exist in which the amino acid sequence of the  $\alpha$  chains is different. For example, Type II collagen consisting of three identical  $\alpha$  chains is designated  $[\alpha_1(II)]_3$ .

Covalently-linked dimers of  $\alpha$  chains are referred to as  $\beta$ -components;  $\beta_{11}$  designates an  $\alpha_1 - \alpha_1$  dimer,  $\beta_{12}$  an  $\alpha_1 - \alpha_2$  dimer etc. Covalently-linked trimers are referred to as  $\gamma$  components.

Gelatin is a term used to indicate a denatured or unfolded collagen; intact or fragmented  $\alpha$  chains,  $\beta$  components and  $\gamma$  components can all be present and referred to as gelatin.

Pro-collagen is the biosynthetic precursor of collagen, consisting of three pro- $\alpha$  chains. The pro-collagen molecule consists of a triple helical region formed by the  $\alpha$  chains and two domains, unique to the precursor, located at the amino- and carboxy-terminal regions of the chains. Intermediates between pro-collagen molecules and collagen molecules in which the extensions are partly removed are termed p-collagens; pn-collagen molecules have the amino terminal extension but not the carboxy-terminal



domain, whereas pc-collagen molecules have the carboxy-terminal extension but no amino-terminal domain.

## 2.2. DIFFERENT COLLAGEN TYPES

Prior to 1970, all vertebrate collagens were regarded as a simple class of molecules composed of two  $\alpha 1$  chains and one  $\alpha 2$  chain with only minor heterogeneity in composition between species (Asghar and Henrickson, 1982). This has, however, changed over the last fifteen years with the discovery of 11 genetically distinct types of collagen, with the possibility of more being characterised in the future. The molecular species associated with each type of collagen all fulfil the criteria for designation as a collagen molecule (Miller and Gay, 1987), that is, they have sizeable domains containing the collagen triple helix and they participate in the formation of extracellular aggregates which function primarily as supporting elements.

The different collagen types can be broadly classified into three groups of molecules (Miller and Gay, 1987). Group 1 molecules contain chains with molecular weights greater than 95,000 and are characterised by a 300 nm uninterrupted helical region. Group 2 molecules similarly contain chains with molecular weights

greater than 95,000, but have several helical regions, interrupted by sequences of residues incapable of triple helix formation. Group 3 molecules are composed of chains with molecular weights lower than 95,000. Table 2.1 lists the different collagen types along with the molecular weight of each chain, in the pro-collagen and collagen forms, and the molecular species formed by the various chains.

### Group 1 Molecules

Type I collagen is found in several major tissues and is by far the predominant collagen in vertebrate organisms. The most common molecular species of this collagen is composed of two identical  $\alpha 1(I)$  chains and one  $\alpha 2(I)$  chain. The three chains are associated intracellularly through specific interactions of the pro- $\alpha$  chains (Kivirikko and Myllyla, 1984). Correct chain alignment is probably achieved in the C-terminal pro-peptide region where the chains are disulphide-linked prior to helix formation (for reviews see Fessler and Fessler, 1978 and Prockop *et al.*, 1979), and during biosynthesis fold, probably from the C-terminus, at a rate limited by the *cis-trans* isomerisation of peptide bonds (Bruckner *et al.*, 1981). Extracellular processing of the pro-collagen removes the non-helical domains of the pro- $\alpha$  chains so that, in the fibrils, the collagen molecules exist almost entirely in the triple helical conformation. In

Table 2.1. Molecular weights and type of  $\alpha$  chain associated with the different collagen types.

GROUP	Collagen	Chains	Chain $M_r$		Molecular species
			Procollagen	Collagen	
1	Type I	$\alpha 1(I)$	140,000	95,000	$[\alpha 1(I)]_2\alpha 2(I)$
		$\alpha 2(I)$	125,000	95,000	$[\alpha 1(I)]_3$
	Type II	$\alpha 1(II)$	140,000	95,000	$[\alpha 1(II)]_3$
	Type III	$\alpha 1(III)$	140,000	95,000–110,000	$[\alpha 1(III)]_3$
	Type V	$\alpha 1(V)$	240,000	115,000	$[\alpha 1(V)]_2\alpha 2(V)$
		$\alpha 2(V)$	160,000	125,000	$[\alpha 1(V)]_3$
		$\alpha 3(V)$	?	?	$[\alpha 1(V)\alpha 2(V)\alpha 3(V)]$
	Type K	$1\alpha$	?	?	?
		$2\alpha$	?	?	
		$3\alpha$	?	?	
2	Type IV	$\alpha 1(IV)$	185,000	185,000	$[\alpha 1(IV)]_2\alpha 2(IV)$
		$\alpha 2(IV)$	170,000	170,000	$[\alpha 1(IV)]_3$
	Type VI	$\alpha 1(VI)$		140,000	$[\alpha 1(VI)\alpha 2(VI)\alpha 3(VI)]$
		$\alpha 2(VI)$	(240,000)	140,000	$[\alpha 1(VI)]_3$
		$\alpha 3(VI)$		140,000	
	Type VII	$\alpha 1(VII)$	?	>170,000	$[\alpha 1(VII)]_3$
3	Type VIII	?	(180,000)	(180,000)	?
	Type IX	$\alpha 1(IX)$	~80,000	~70,000	$[\alpha 1(IX)\alpha 2(IX)\alpha 3(IX)]$
		$\alpha 2(IX)$	~80,000	~70,000	
		$\alpha 3(IX)$	~80,000	~70,000	
	Type X	$\alpha 1(X)$	59,000	49,000	$[\alpha 1(X)]_3$



addition to heterotrimers, homotrimers composed of three  $\alpha 1(I)$  chains are formed in some instances.

Type II collagen is found as fibrous elements in tissues such as hyaline cartilages, the notochord, nucleous pulposus and vitreous humour. Assembly through pro- $\alpha$  chains and extracellular processing occurs as in type I collagen. Type II molecules are homotrimers consisting of three  $\alpha 1(II)$  chains.

Fibrils formed from type III collagen molecules are generally found together with the larger type I collagen fibres in connective tissues. Type III collagen contains three identical  $\alpha 1(III)$  chains (Chung and Miller, 1974; Chung *et al*, 1974). Unlike type I and type II pro-collagen, however, extracellular processing of type III pro-collagen often results in the retention of the N-terminal pro-peptide sequences (Kivirikko and Myllyla, 1984). In some instances, the N-terminal pro-peptide is also retained in the fibrillar aggregates and this may explain the smaller size of type III fibrils compared to the fibrils of type I.

Type V collagen is found in a number of tissues but only ever represents a small fraction of the total collagen present (Miller, 1984). The heterotrimer composed of two  $\alpha 1(V)$  chains and one  $\alpha 2(V)$  chain is most prevalent, but other heterotrimers and homotrimers

(see Table 2.1) have also been isolated.

Type K collagen, like type V collagen, is only a minor collagen and its occurrence is limited to cartilaginous structures. Preparations of type K collagen contain three identifiable chains (1a, 2a and 3a) but there is a lack of data concerning the nature and organisation of the chains (Miller and Gay, 1987).

The essential features of the group 1 pro- $\alpha$  chains can be represented as in Fig. 2.1a. Firstly, there is an N-terminal pro-peptide (pn) domain in which the initial non-helical domain is followed by a short helical region and then a short non-helical segment. Secondly, there is an uninterrupted helical domain of approximately 300 nm in which the chain has the Gly-X-Y triplet repeating sequence characteristic of the collagen triple helix; this is flanked at both ends by short non-helical sequences known as teleopeptides. Finally, there is the C-terminal pro-peptide (pc) part of the chain. In general, the non-helical sequences are characterised by the presence of acidic and large hydrophobic amino acid residues; there are also several cysteinyl residues which are involved in intra-chain disulphide bonds in the pN domain, and in inter-chain disulphide bonds in the pC domain. There can be significant sequence variation in the helical regions of different  $\alpha$  chains but the triplet sequence Gly-X-Y is invariably

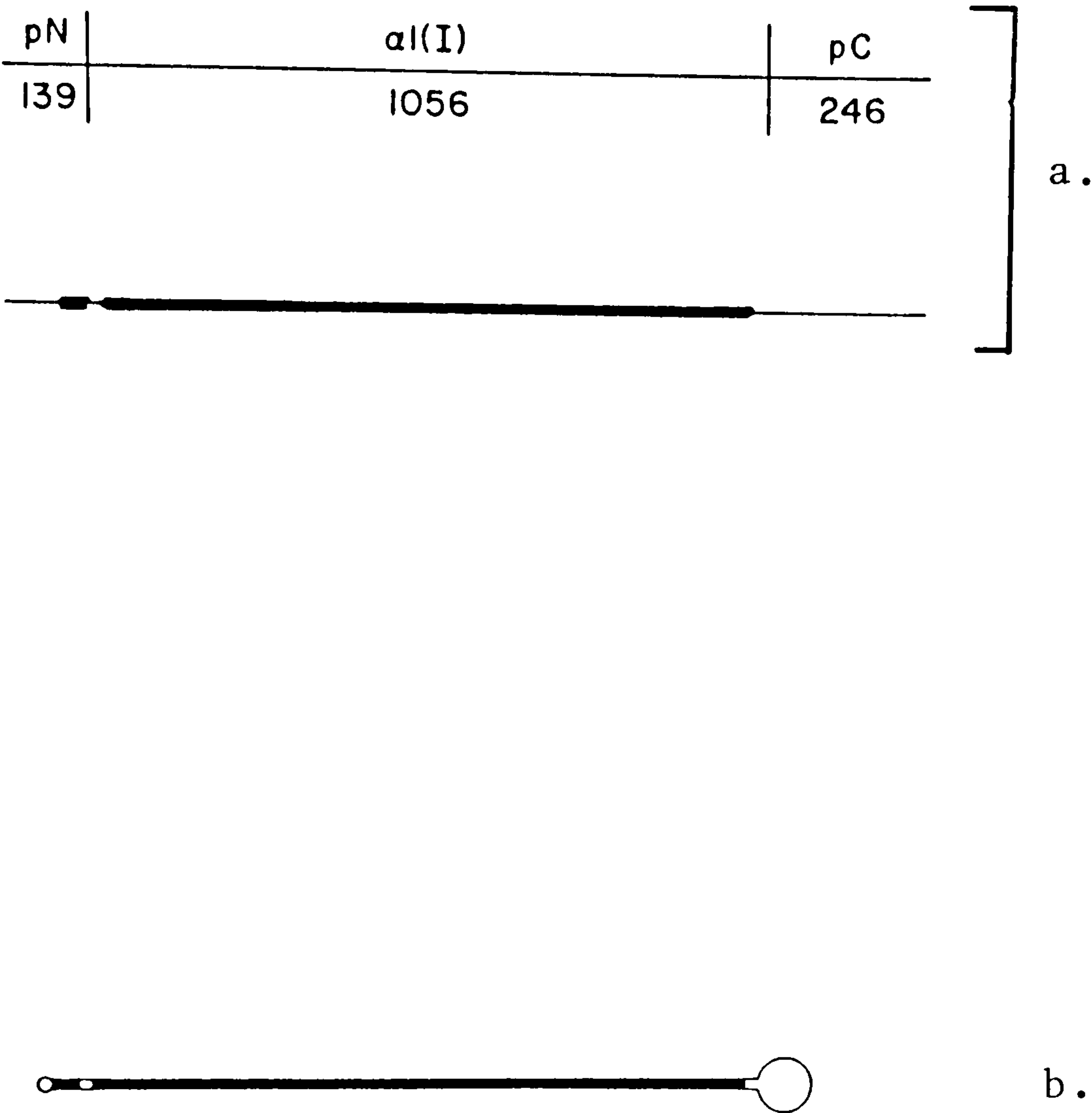


Figure 2.1. Schematic diagram of the chain and molecular parameters of group 1 collagen molecules:  
a) the domain structure of pro- $\alpha 1(I)$  with the numbers denoting the amino acid residues in each domain and underneath regions of triplet (heavy lines) and non-triplet (lines) along the chain;  
b) the conformation of a procollagen molecule with helical (solid areas) and globular or non-helical regions (open areas) illustrated.  
(From Miller and Gay, 1987).



maintained, emphasising the importance of the positioning of a glycyl residue in every third position in triple helical domains. The Group 1 pro-collagen molecules reflect the structural features of the individual pro- $\alpha$  chains (Fig. 2.1b) and consist essentially of two globular domains, bracketing a lengthy triple helical region which is interrupted, for a short span, in the N-terminal portion of the molecule.

Following extracellular processing, the aggregates of types I, II and III collagens exist in fibrils in which the molecules associate laterally but exhibit definitive axial displacements, being staggered relative to one another by integral multiples of the distance,  $D$ , 67 nm. The overall length of each molecule is approximately  $4.5D$  (300 nm) and axial displacement of the molecules thus results in alternating overlap and gap zones within the fibrils (Fig. 2.2). The sequence in which extracellular processing occurs (i.e. the order of removal of the pc and pn domains) may be a controlling factor in the ultimate size and morphology of the fibrils formed (Davidson *et al.*, 1975; Fleischmajer *et al.*, 1981; Fleischmajer *et al.*, 1983; Miyahara *et al.*, 1984).

In certain tissues of young organisms, a delay in the formation of crosslinks between collagen molecules in the fibres allows native collagen monomers to be

extracted under mild aqueous conditions (Ramachandran, 1967). Much of the work carried out on collagen in the past has been performed on type I collagen extracted in this manner.

### Group 2 Molecules

Type IV collagen occurs exclusively in basement membrane structures. Aggregates of type IV collagen are not readily dissociated, due to lysine-derived crosslinks and probably covalent interactions with other constituents of basement membranes (Miller and Gay, 1987). There is also evidence that disulphide crosslinks are involved in stabilising the aggregates (Kleinman *et al.*, 1982). The number of different  $\alpha$  chains in type IV collagen is not known for certain, but the molecular species  $[\alpha 1(\text{IV})]_2 \alpha 2(\text{IV})$  and  $[\alpha 1(\text{IV})]_3$  have been identified.

On exposure of type IV aggregates to pepsin under conditions which cleave non-helical regions, considerable fragmentation occurs, indicating that there are numerous regions within type IV collagen lacking the typical collagen fold. The general structural features of the type IV molecule have been discerned by electron microscopy. Rotary shadowing of molecules extracted from mouse tumour revealed the presence of highly flexible thread-like molecules, terminating at one end in a globular knot (NC1 domain)

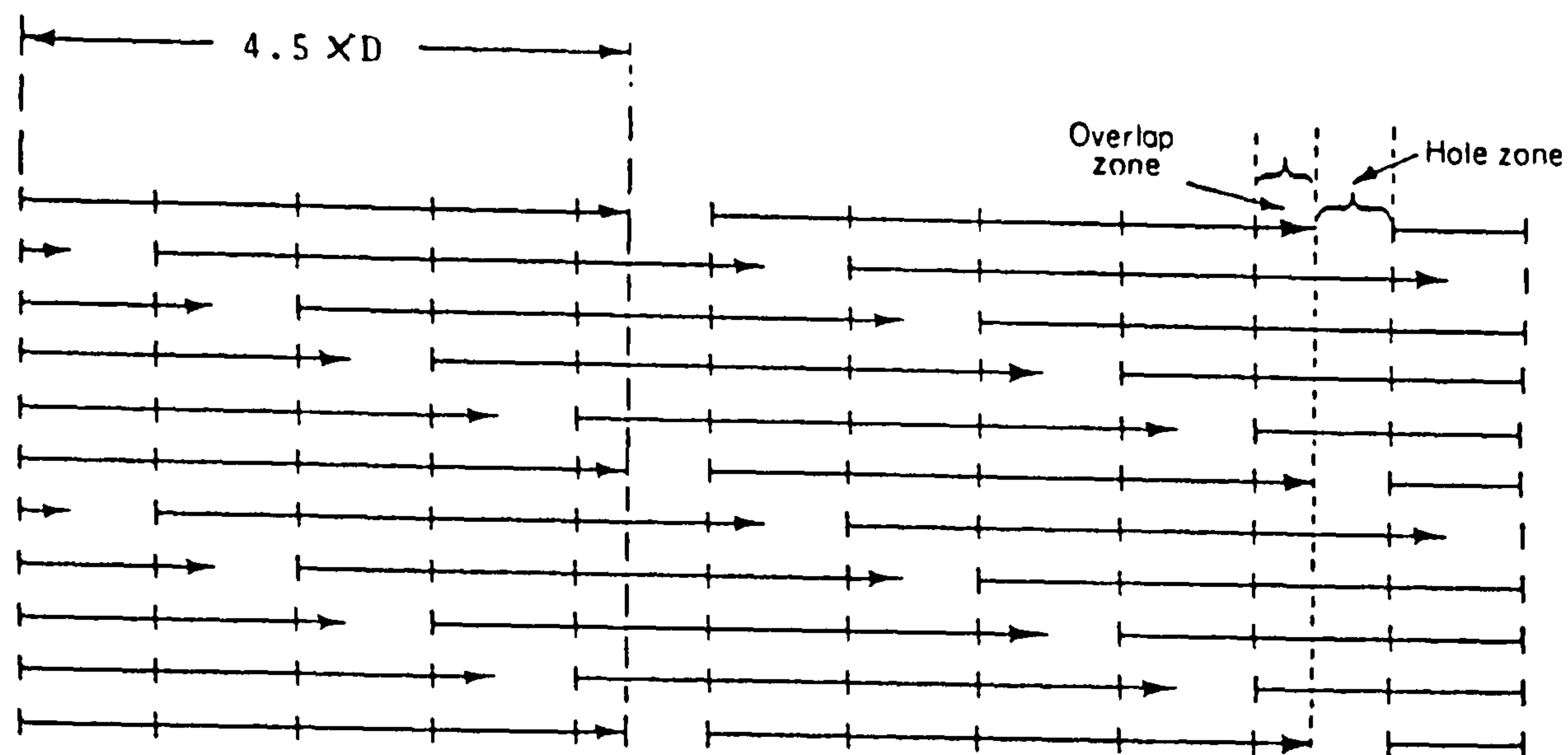


Figure 2.2. Illustration of the overlap and gap zones created in collagen fibres as a result of the axial displacement of the molecules.

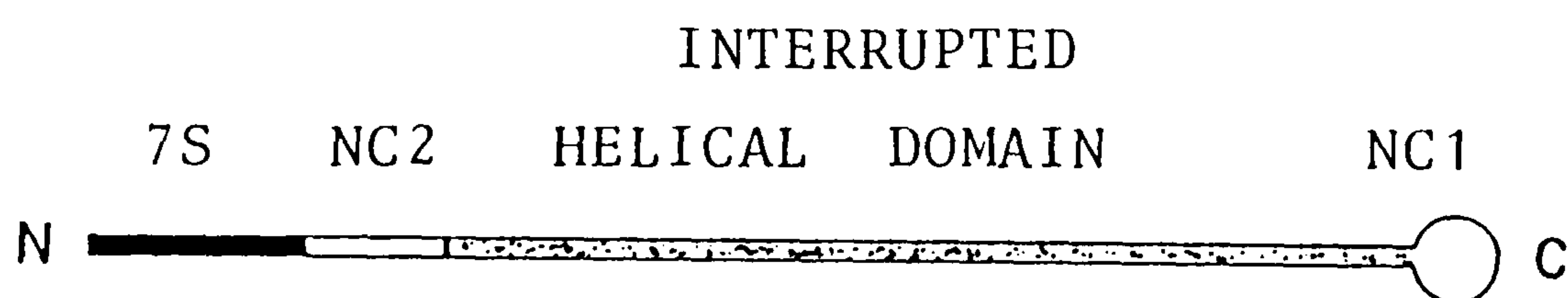


Figure 2.3. Schematic illustration of the domain structure of type IV collagen showing the positions of the NC1, NC2, 7S and interrupted helical domains.



and at the other end in a compact helical zone (7S domain) (Hoffman *et al.*, 1984; Timpl *et al.*, 1981). Next to the 7S domain is a relatively large non-helical region (NC2 domain), followed by a long helical region which has 12 interruptions of the Gly-X-Y triplet structure, giving the molecule the high degree of flexibility (Fig. 2.3).

Aggregation of the type IV molecules appears to be through formation of tetramers, by interaction of the 7S domains; the tetramers then form a mesh-like network, suitable to serve as the scaffold for sheaths of basement membrane through interaction of the globular domains.

Type VI collagen contains three different  $\alpha$  chains,  $\alpha 1(\text{VI})$ ,  $\alpha 2(\text{VI})$  and  $\alpha 3(\text{VI})$ , the molecular species being a heterotrimer of all three chains. There is also evidence for a homotrimer composed solely of the more acidic  $\alpha 1(\text{VI})$  chain (Furuto and Miller, 1981). The essential features of the type VI molecule are a central triple helical domain, of approximately 105 nm length, terminating in large globular domains at both ends (Furthmayr *et al.*, 1983). The fibrillar structure of type VI collagen involves the end-to-end association of tetrameric molecules composed of intertwined dimer subunits (Miller and Gay, 1987).

There is very little data on the chemical properties, chain composition and domain structure of type VII and type VIII collagen. Type VII collagen appears to be constructed from three identical  $\alpha 1(\text{VII})$  chains, whereas the number of different  $\alpha$  chains present in type VIII collagen is not known (Miller and Gay, 1987). Both type VII and type VIII collagen, however, have interrupted helical domains and are thus classed as group 2 molecules.

### Group 3 molecules

Group 3 molecules differ from group 1 and group 2 molecules as they are composed of chains with molecular weights less than 95,000. Type IX and type X collagen are the most well characterised of group 3 molecules and both occur exclusively in cartilage.

Type IX collagen molecules consist of three different  $\alpha$  chains, the molecular species being designated  $[\alpha 1(\text{IX})\alpha 2(\text{IX})\alpha 3(\text{IX})]$ . Each pro- $\alpha$  chain has an approximate molecular weight of 80,000, the separate  $\alpha$  chains having a molecular weight of 70,000. The collagen IX molecules have a central triple helical region flanked by two interrupted helical regions which are attached to small pN and pC domains (Miller and Gay, 1987).

Type X collagen is probably composed of three identical  $\alpha$  chains, although this is not known for certain. The molecule has a triple helical domain, approximately 150 nm long, with a globular domain at one end of the helix (Schmid *et al.*, 1984). It is not known whether the globular domain is at the N-terminal or C-terminal end of the molecule.

To summarise, it has been shown that collagen molecules and fibres are much more complex than initially envisaged. However, type I collagen is by far the most common, and as such is of course undoubtedly the collagen type which has most influence on the type of gelatin obtained industrially. The basic triple helical structure which occurs in all the collagens is of paramount importance in dictating their properties; the conformation and stabilisation of the triple helix will now be discussed in more detail.

## 2.3. THE COLLAGEN TRIPLE HELIX AND ITS STABILISATION

### 2.3.1. Triple Helical Structure

The helical regions of collagen molecules have a characteristic amino acid composition, one third of which is glycine and up to one third the imino acids,



proline and hydroxyproline. This is quite different from the composition of other proteins and, indeed, hydroxyproline occurs almost exclusively in collagens. Within the helical regions of the chains, the sequence of amino acid residues is composed of Gly-X-Y repeating triplets, with proline often occurring in position 2 and hydroxyproline in position 3 of the triplets (Hannig and Nordwig, 1967).

The present knowledge of the nature of the helical secondary structure of collagen molecules was derived from detailed X-ray diffraction studies and interpreted in the mid 1950's in the light of the work of Pauling and Corey (1951) on bond angles and lengths of the peptide unit. The X-ray fibre diffraction was generally carried out on collagen fibres reprecipitated in various forms from solutions of soluble monomeric collagen, obtained from the tissues of young animals prior to molecular crosslinking.

The basic features of the wide angle X-ray diffraction are a strong meridional arc at  $2.86\text{\AA}$ , arising from the repeat distance along the fibre axis and an  $11\text{\AA}$ , hydration-sensitive, equatorial reflection relating to distances transverse to the fibre axis; further arcs can also be seen at  $9.5\text{\AA}$  and  $4\text{\AA}$  (For further information on the X-ray data, see the reviews of Bear, 1952; Harrington and Von Hippel, 1962; Low, 1953). The generally accepted interpretation of the fibre

diffraction arose from the work of Ramachandran (Ramachandran, 1956; Ramachandran and Ambady, 1954; Ramachandran and Kartha, 1954; Ramachandran and Kartha, 1955) who proposed a model for collagen structure using the following assumptions:

- 1) the N-H and C=O bonds of the protein backbone are at right angles to the fibre axis (Ambrose and Elliot, 1951).
- 2) the helix is a left-handed helix as the sign of the specific rotation is opposite to that of the  $\alpha$  helix.
- 3) all peptide bonds are in the *trans* configuration as indicated by infra red studies.

Similar structures were proposed at the same time by two other groups of workers (Rich and Crick, 1955; Cowan and McGavin, 1955) and it is upon these proposals that the present model for collagen is based.

The proposed model can be envisaged by considering three polypeptide chains, each coiled in a left-handed helix with ten residues in three turns, arranged parallel to each other in an equilateral triangle of side 5Å (see Fig. 2.4a). The three chains are then further twisted about a common central axis to give a slightly right-handed super helix with an overall



repeat distance of approximately 86Å and a translation of 2.86Å from a residue on one chain to the corresponding residue on another, thus accounting for the strong meridional arc. As a consequence of the right-handed twist about the common central axis, each chain in the resultant structure has a repeating unit consisting of three residues (see Fig. 2.4b). Initially, Ramachandran had proposed an exact three-fold screw symmetry for each chain, the three chains packing together in a linear parallel fashion (Ramachandran and Kartha, 1954). This was modified, as described above, to take into account the revised number of residues per turn, but it is interesting to note that the exact 3-fold screw axis was found to be correct for the polypeptides, poly-L-proline (Cowan and McGavin, 1955), polyglycine (Crick and Rich, 1955) and poly-L-hydroxyproline (Sasisekharan, 1959).

### 2.3.2. Stabilisation

Although general agreement was reached on the helical parameters for collagen, there were minor differences between the three groups of workers in their interpretations of the molecular structure of the triple helix. Rich and Crick (1955) described two very similar structures, I and II, which were both compatible with the X-ray data. The differences between structures I and II are in the location of the



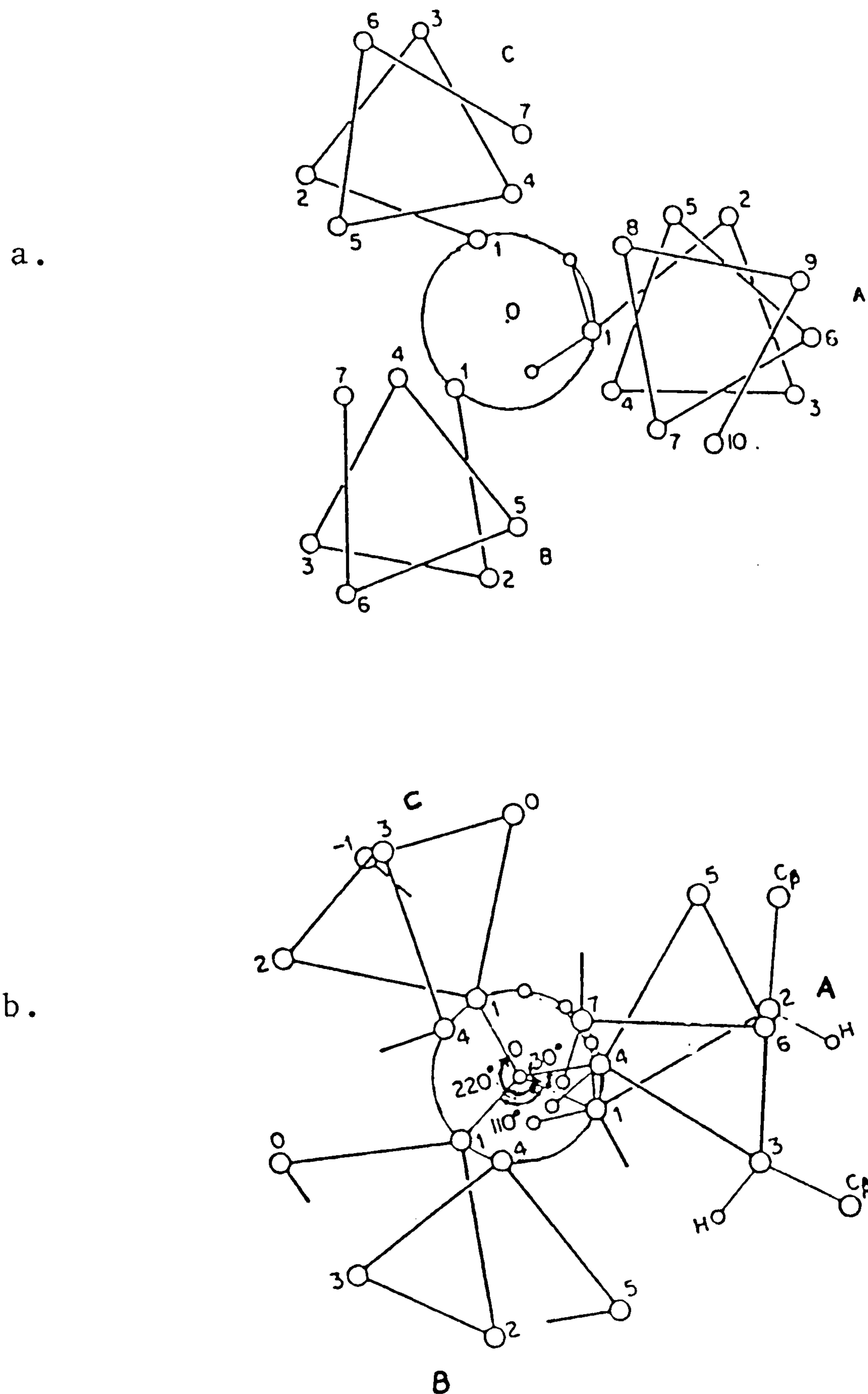


Figure 2.4. a) The three left-handed helices of the collagen structure arranged parallel to each other in an equilateral triangle of side  $5\text{\AA}$ . The  $\alpha$  carbons of each chain are numbered with the repeat structure of ten residues in three turns being illustrated. b) The three left-handed helices are wound about the central axis with a right-handed twist. The value of  $30^\circ$  per three residues of the super coiling twist leads to a major pitch of about  $108\text{\AA}$ , corresponding to 36 residues and a resultant structure about the central axis such that each chain has a repeating unit consisting of three residues.

hydrogen bonds stabilising the helix; the same number of hydrogen bonds are present in both structures, that is, one hydrogen bond per triplet. If the peptide bond which is nearest the centre of the three chains is designated position 1 and the next two groups up the chain 2 and 3 respectively, then structure I involves hydrogen bonds between the N-H of the residue in position 1 and the C=O of the residue in position 1 on the neighbouring chain; in structure II the hydrogen bonds are between the N-H of the residue in position 1 and the C=O on position 2 of the neighbouring chain (see Fig. 2.5). Structure I can accommodate glycine only in positions 1 and 3 of the Gly-X-Y triplet and any residue in position 2, whereas structure II, while only accommodating glycine in position 1, can have any residue, including proline and hydroxyproline, in positions 2 and 3. Structure II was generally favoured on the grounds that it was more sterically favourable in terms of the Gly-Pro-Hyp sequence.

In addition to the structures involving one hydrogen bond per triplet, Ramachandran and Kartha (1955), using Pauling's principle that the maximum number of hydrogen bonds would be formed in a stable structure, proposed the structure shown in Fig. 2.6, containing two hydrogen bonds per triplet. This eliminated the ambiguity of the two singly-bonded structures. Again, the two-bonded model is capable of accommodating the Gly-Pro-Hyp sequence, glycine in position 1 being a







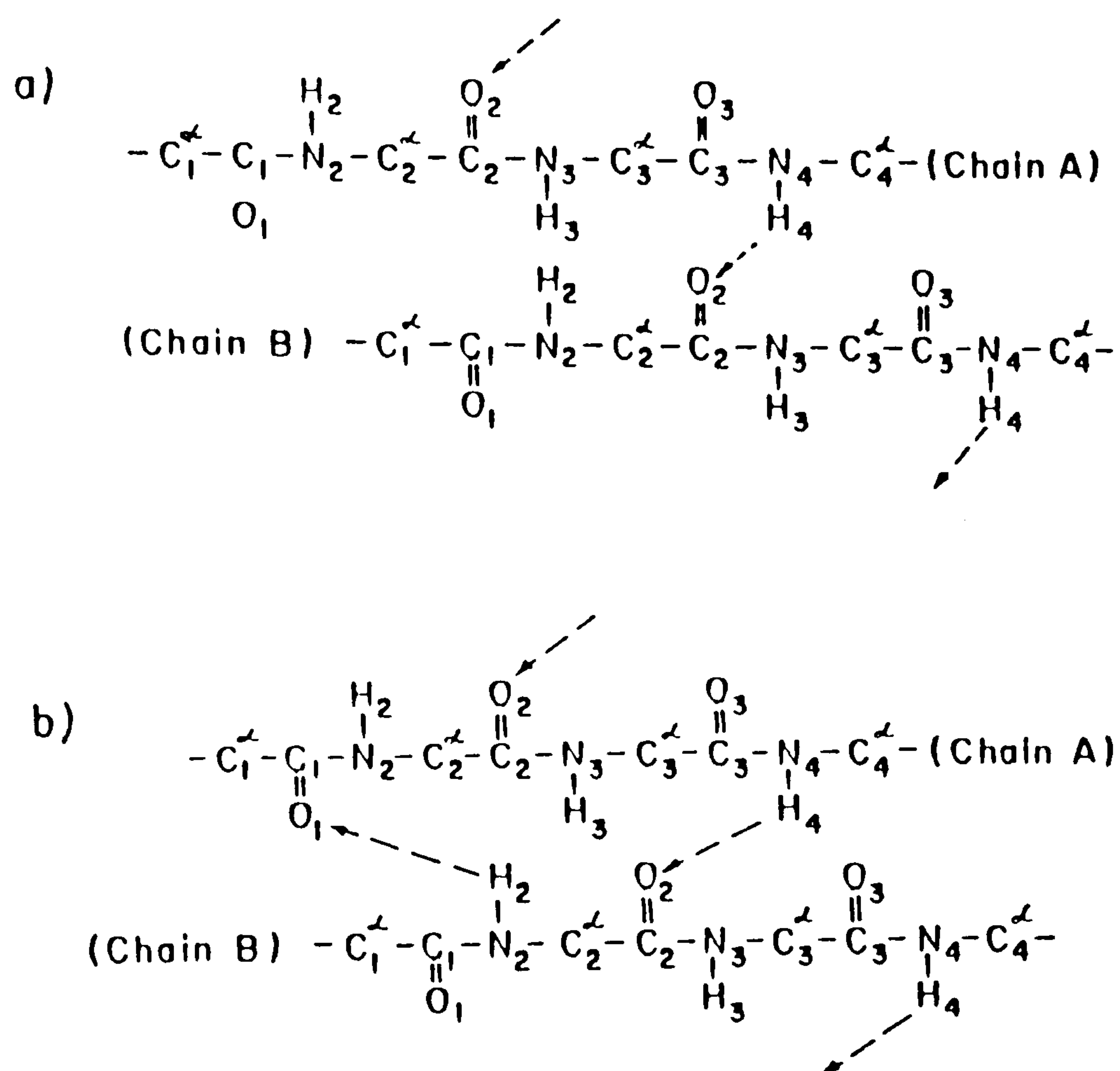


Figure 2.7. Schematic diagram of the internal hydrogen-bonded structures proposed for collagen a) one-bonded model; b) two-bonded model.

necessity. Fig. 2.7 shows schematically the hydrogen bonds involved in the one- and two-bonded models.

There were numerous attempts to determine experimentally the number of hydrogen bonds in soluble collagen by the method of hydrogen exchange; however, the results were often contradictory. According to some studies, there was one stable hydrogen bond per triplet (Bradbury, *et al.*, 1958; Traub *et al.*, 1969; Katz, 1970; Esipova *et al.*, 1972), whereas results of other workers indicated two hydrogen bonds per triplet (Bensusan and Nielsen, 1964; Jordan and Speakman, 1965; McBride and Harrington, 1967a,b; Kingham and Brisbin, 1968). The contradictory results were explained by Yee *et al.* (1974) who showed that there were two classes of exchangeable hydrogens in collagen, slowly and very slowly exchangeable, and by Privalov *et al.* (1979) who demonstrated a difference in the temperature-sensitivity of the two classes, the slowly exchangeable hydrogens becoming rapidly exchangeable at higher temperatures. It has been found that the number of exchangeable hydrogens in each class is the same for collagens of different composition, stability and enthalpy of melting, being  $(1.0 \pm 0.1)$  very slowly and  $(0.7 \pm 0.1)$  slowly exchangeable hydrogens per triplet.

Assignment of the very slowly exchangeable hydrogens to the hydrogens involved in the bonds at the first position in the triplets and the slowly exchangeable hydrogens to the bonds in the second position of the triplets (Fig. 2.7) thus seems to support Ramachandran's two-bonded model, particularly as the number of bonds in the second position would be less than one per triplet, since imino acids in this position, having no hydrogen on the nitrogen, would be incapable of forming a hydrogen bond. The situation was not as simple as this, however, as the hydrogen bond between the two groups in the second position would be too long (5.1Å) (Ramachandran and Chandrasekharan, 1968) and it has been suggested that a water molecule, or possibly two, act as bridges between the C=O and N-H groups (Ramachandran and Ramakrishnan, 1976).

The stability of collagen molecules in solution also reflects the type of collagen and the species from which it was obtained. On heating a solution of collagen, there is a sharp change in physical properties such as viscosity and optical behaviour at a specific transition temperature where the triple helical structure is lost and replaced by a random coil-like conformation; the temperature at which this happens is known as the melting temperature,  $T_m$ . The melting temperatures of collagens from different sources are dependent on the collagen imino acid



composition (Privalov, 1982) and as the transition enthalpy,  $\Delta H_m$  was found to correlate well with  $T_m$  (Fig. 2.8) a correlation between  $\Delta H_m$  and imino acid content was also expected; this was found to be the case. (See Fig. 2.9a). However, there is a much better correlation between the transition enthalpies and the hydroxyproline content alone as is shown in Fig. 2.9b.

The special role of hydroxyproline in the stabilisation of collagen structure was first noted in 1953 (Gustavson, 1953) but later it was shown that collagen melting temperature correlated with the total content of proline and hydroxyproline (Burge and Hyness, 1959a, b; Piez and Gross, 1960). The ratio of proline to hydroxyproline for vertebrate collagens is similar, and thus the increased stabilisation due to hydroxyproline was considered to be a reflection of a more general correlation with imino acid content. The increased stability was explained thermodynamically in terms of a decrease in entropy for chains having many rigid pyrrolidine rings. Consider the melting of a collagen triple helix: let  $\Delta G$ ,  $\Delta H$  and  $\Delta S$  be the differences in free energy, enthalpy and entropy between the ordered and disordered conformation. At the transition midpoint  $\Delta G = 0$  and  $T = T_m$

$$\text{i.e.} \quad \Delta G_m = \Delta H_m - T_m \Delta S_m = 0$$

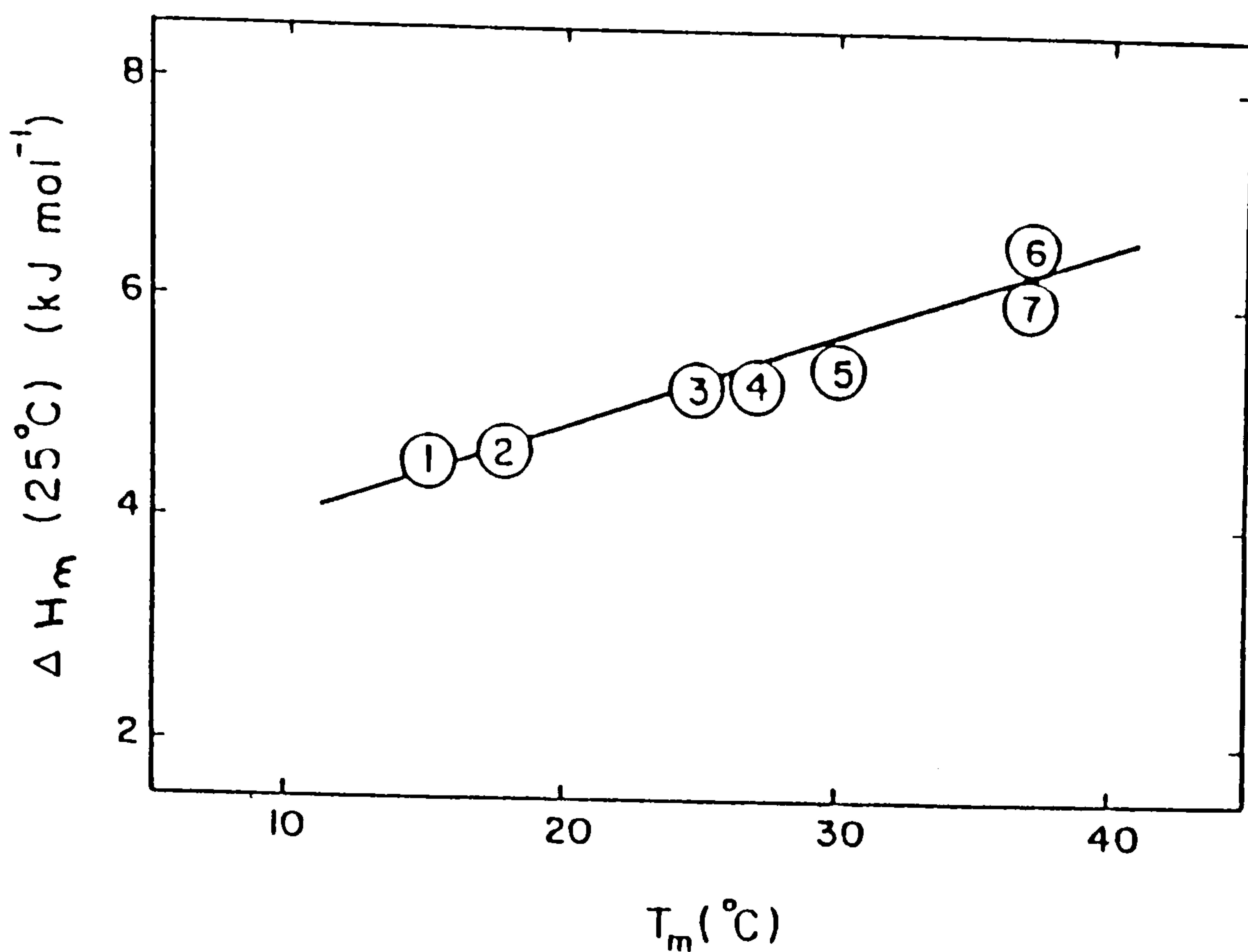


Figure 2.8. Correlation between melting temperature ( $T_m$ ) and enthalpy of melting ( $\Delta H_m$ ) for collagens from different species: (1) cod skin, (2) halibut, (3) frog skin, (4) pike skin, (5) carp swim bladder, (6) rat skin, (7) sheep skin. (From Privalov, 1982).

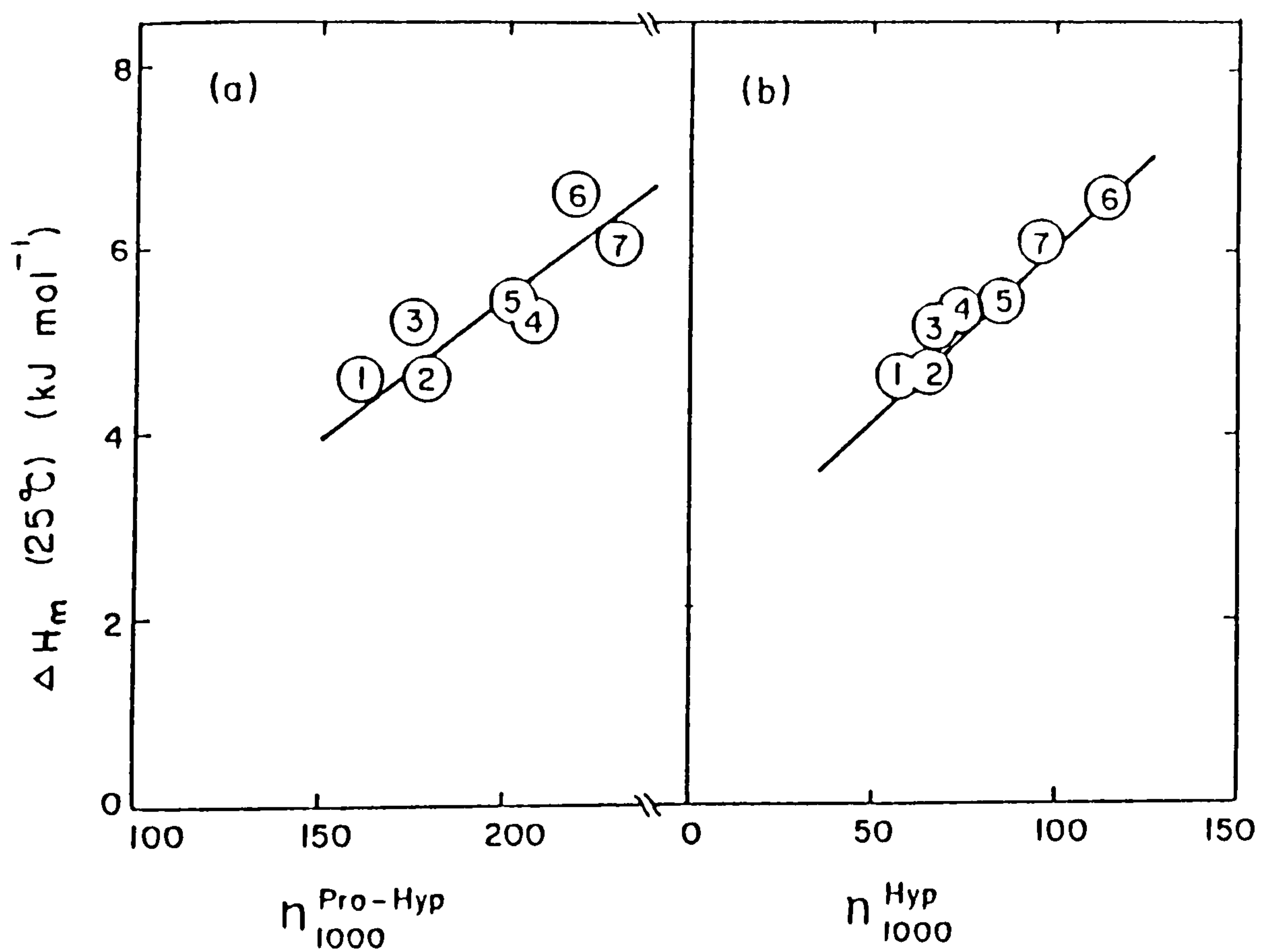


Figure 2.9. Correlation between collagen imino acid content and enthalpy of melting ( $\Delta H_m$ ). (1) - (7) are the same as in Fig. 2.8. a) Total prolyl and hydroxyprolyl content in terms of number of residues per 1000 ( $n_{1000}^{Pro-Hyp}$ ) b) Hydroxyprolyl content only ( $n_{1000}^{Hyp}$ ) (From Privalov, 1982).

therefore  $T_m = \Delta H_m / \Delta S_m$

The change in entropy  $\Delta S_m$  would decrease with the increase in the total number of pyrrolidine groups and therefore higher melting temperatures would be expected for collagens rich in imino acids.

This explanation, however, did not explain the increased enthalpy of melting with increase in imino acid content (Privalov, 1968; Privalov and Tiktopulo, 1970) since proline cannot create any additional bonds in the collagen structure, unlike hydroxyproline which contains an additional OH group capable of hydrogen bonding. It was also shown that collagen-like structures formed by synthetic polypeptides containing hydroxyproline were more stable than those of corresponding polypeptides containing proline (Traub and Piez, 1971; Rao and Adams, 1979; Engel *et al.*, 1977). It therefore appeared that hydroxyproline did have a special role in the stabilisation of collagen structure, and indeed, reinvestigation of the data on collagen thermal stability (Burjanadze, 1979) revealed that  $T_m$  correlated much better with hydroxyproline content than with total imino acid content.

Invertebrate collagens show no obvious correlation between hydroxyproline content and  $T_m$ . This has been explained by the occurrence of hydroxyproline with



equal preference in the second and third positions of the Gly-X-Y triplet in invertebrates (Goldstein and Adams, 1968, 1970), while in vertebrates, hydroxyproline is located exclusively in the third position of the triplet (Piez, 1976 and Fietzek *et al.*, 1979). Further studies on collagen (Gryder *et al.*, 1975; Gelman *et al.*, 1976) have shown that 3-hydroxyproline located in the third position of the Gly-X-Y triplet does not have the same stabilising effect as 4-hydroxyproline, and it has therefore been concluded that 4-hydroxyproline in the third position of the triplet has a major role in triple helix stabilisation (Privalov, 1982).

Ramachandran *et al.* (1973) proposed a model in which hydroxyproline was involved in structure stabilisation (Fig. 2.10b); (see also Ramachandran and Ramakrishnan, 1976). In this model, the water bridge mentioned previously (illustrated in Fig. 2.10a) can form one extra hydrogen bond with the oxygen of hydroxyproline if the hydroxyproline occurs at the third position in the triplet. A similar model proposed by Fraser *et al.* (1979) assumed that **hydroxyproline** and glycine were connected by a bridge of two water molecules. The involvement of water in structure stabilisation through water bridges is not improbable as it is well established that water molecules are capable of interacting with their neighbours, and it is possible that hydroxyproline may act as an initiator for an

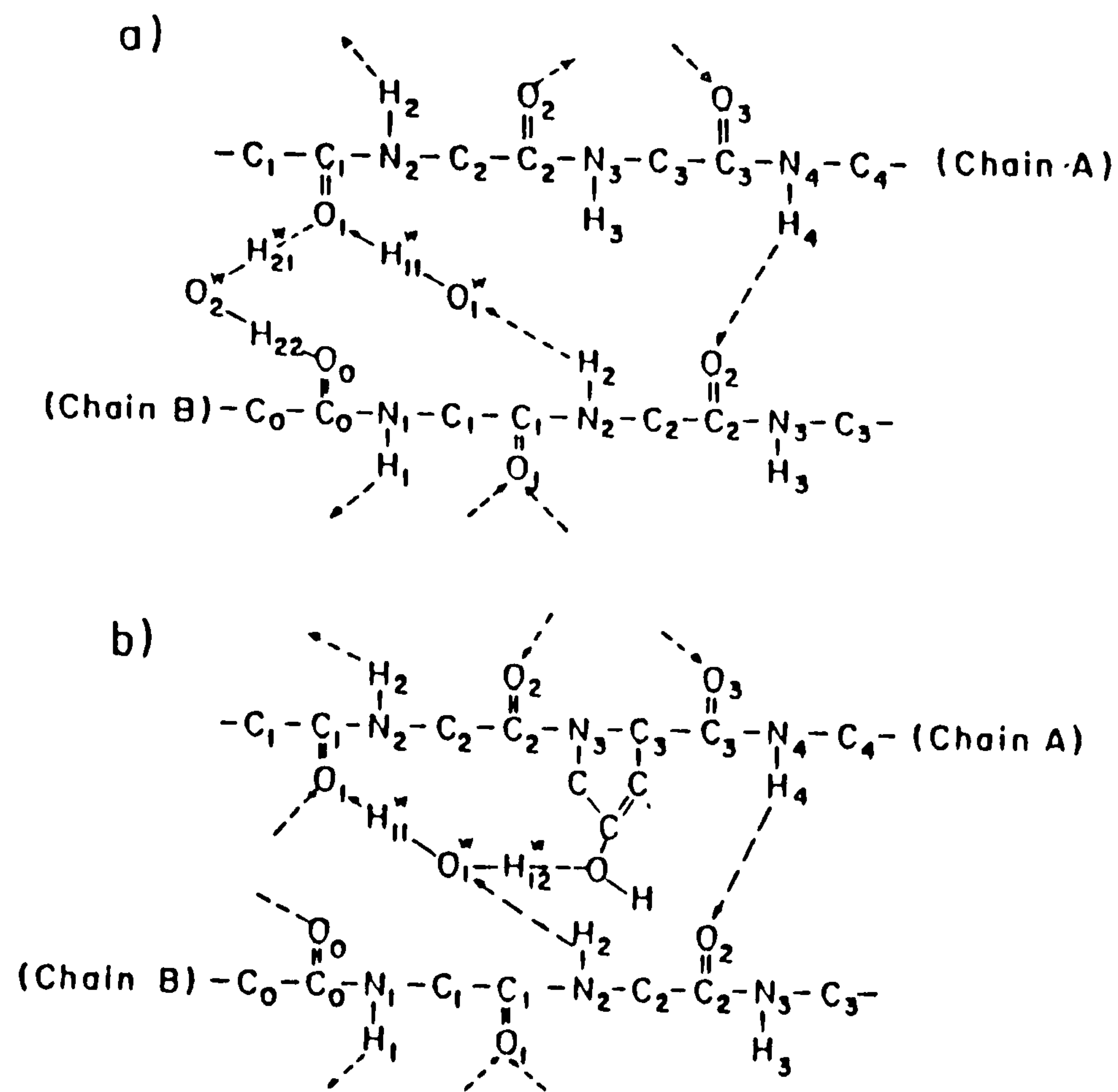


Figure 2.10. Schematic diagrams of hydrogen-bonded networks involving water ; a) with no hydroxyproline present and b) with hydroxyproline involved in the hydrogen bonding. (From Ramachandran and Ramakrishnan, 1976).

extensive network of hydrogen-bonded water, enveloping the collagen molecule. On the basis of this, several theoretically possible models were proposed involving water bridges between different parts of the collagen molecule (See Tumanyan, 1970; Esipova and Tumanyan, 1973 and Tumanyan and Esipova, 1973) and it is now evident that the stability of collagen must be due, in part, to specific incorporation of water molecules.

There is a great deal of evidence indicating the crucial role of water in maintaining the collagen structure. X-ray diffraction studies (Rougvie and Bear, 1953; Esipova *et al.*, 1958; Esipova and Chirgadze, 1969; Luscher *et al.*, 1973) and infra red studies (Chirgadze *et al.*, 1969; Susi *et al.*, 1971) both showed that collagen loses its regular structure when water is removed.

The water molecules are not all bound to the same extent, and from the analysis of sorption isotherms it was concluded that there were 2 or 3 strong water-binding sites per triplet in collagen.

X-ray evidence indicates the strongly bound water molecules are located at a distance of 3Å from the collagen molecular axis (Esipova *et al.*, 1958), and infra red dichroism studies (Fraser and MacRae, 1959; Suzuki and Fraser, 1974) and nuclear magnetic resonance studies (Berendsen, 1972; Berendsen and Mighelsen,



1965; Dehl and Hoeve, 1969; Chapman and McLaughlan, 1969; Chapman *et al.*, 1971; Fung and Siegel, 1972; Mighelsen and Berendsen, 1973) suggest that the water molecules are preferentially orientated to the fibre axis.

From this discussion, it is clear that although the general stereochemical features of the collagen triple helix are in no doubt, the actual stabilisation of the structure is very complex, involving numerous molecular interactions, the most important of which are the inter-chain hydrogen bonds of the peptide backbone, the specific role of 4-hydroxyproline in the third position of the triplet and additional stabilisation through collagen-water interactions.

#### 2.4. EXTRACTION OF COLLAGEN/GELATIN

Collagen is converted into gelatin by denaturation, coupled with physical and chemical degradation, of intact collagen molecules. This process produces solutions of polypeptide chains with approximately the same amino acid composition as the parent collagen, which on cooling are capable of forming a gel and, as such, have been of great importance in the food and photographic industries.

The bulk of collagen molecules in connective tissues are covalently crosslinked through aldimine and aldol formation via the  $\epsilon$ -amino group of lysine and are therefore insoluble (Light and Bailey, 1982). However, as a result of the delay in intermolecular crosslink formation, young tissues frequently have collagen monomers present which can be extracted with cold, dilute, neutral salt or acid solutions (Ramachandran, 1967). A solution of collagen monomers is highly viscous and has a large negative specific optical rotation due to the rigid long molecules and triple helical conformation.

The simplest example of the collagen to gelatin transition is the disruption of non-covalent bonds by thermal denaturation of soluble collagen. Thermal denaturation occurs over a narrow temperature range, close to 40°C for solutions of mammalian collagens, and is accompanied by an abrupt change in intrinsic viscosity, specific optical rotation, sedimentation and light scattering properties. These changes in physical properties are a result of the conformational transition from the highly extended triple helix to disordered, flexible random coils (gelatin). Fig. 2.11 shows schematically the conversion of type I soluble collagen to gelatin.



Commercial gelatins are not derived from soluble collagen but from the insoluble, crosslinked fibrils of bone and skin collagen. Industrial processes employ lyotropic and hydrolytic treatments with aqueous solutions of acid or base to break the covalent bonds and solubilize the polypeptide chains. The type of gelatin finally obtained depends on the combination of raw materials, extent and type of raw stock treatment (pretreatment) and extraction conditions.

### Raw materials

The principal source of collagen for gelatin production is the skin and bone of cattle and pigs, although other mammalian species and fish are also used, particularly for research. Prior to pretreatment, hides are washed free of debris and preservatives, dehaired, degreased and may also be chopped to facilitate handling. In North America, pigskins are a particularly important source of raw stock for edible gelatin. Cattle and oxen are the major source of bone raw stock. The bone is first crushed to the desired size (ca. 1-1.5 cm) and degreased. The principal non-collagen component of bone, the mineral hydroxy-apatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , is then removed by leaching the bone in dilute acid (usually 5% HCl) for a few weeks; the resulting material is called ossein (Rose, 1986). The ossein is washed neutral and kept moist until the pretreatment stage.



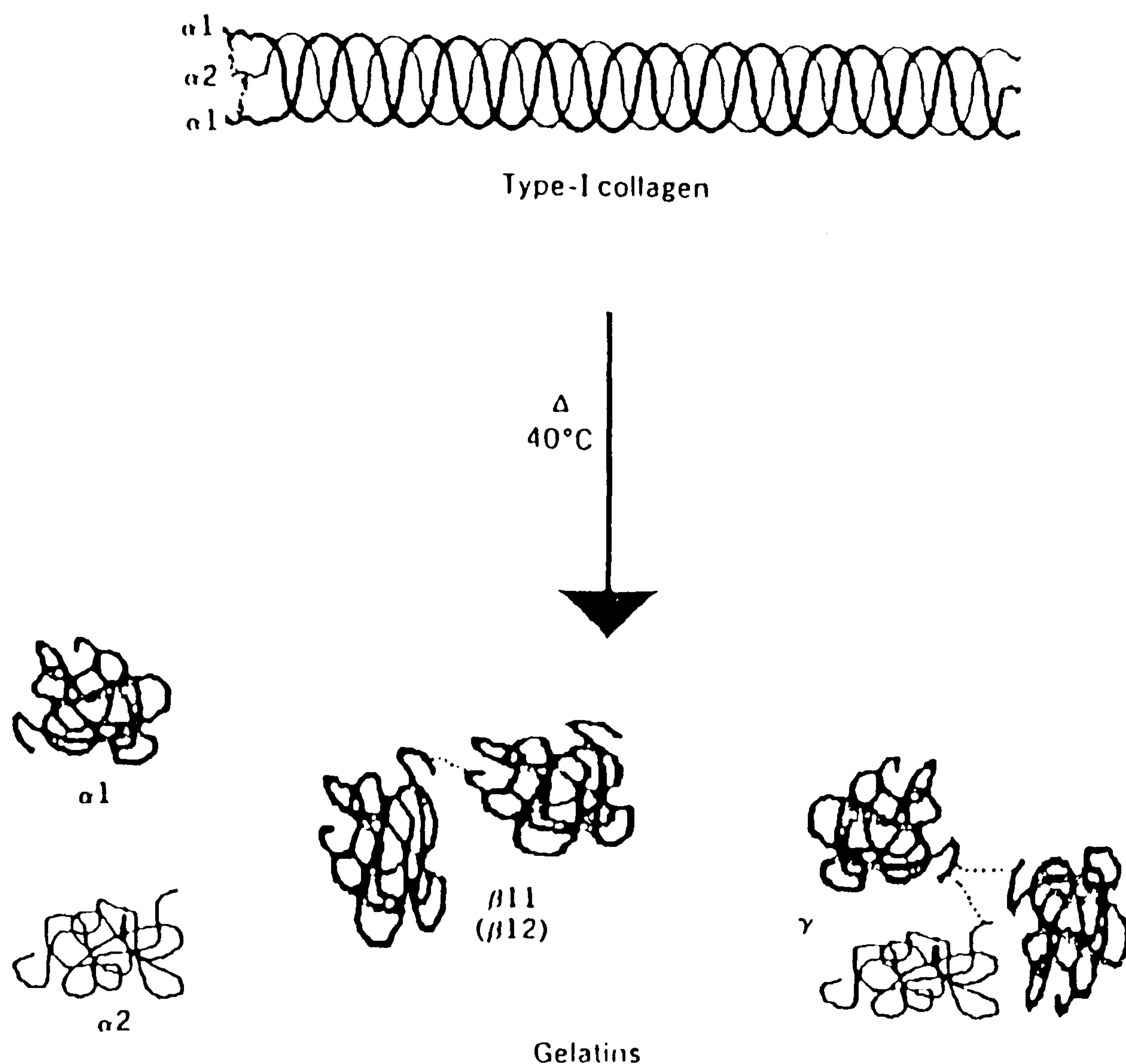


Figure 2.11. The thermal denaturation of type I soluble collagen showing the different denaturation products obtained.

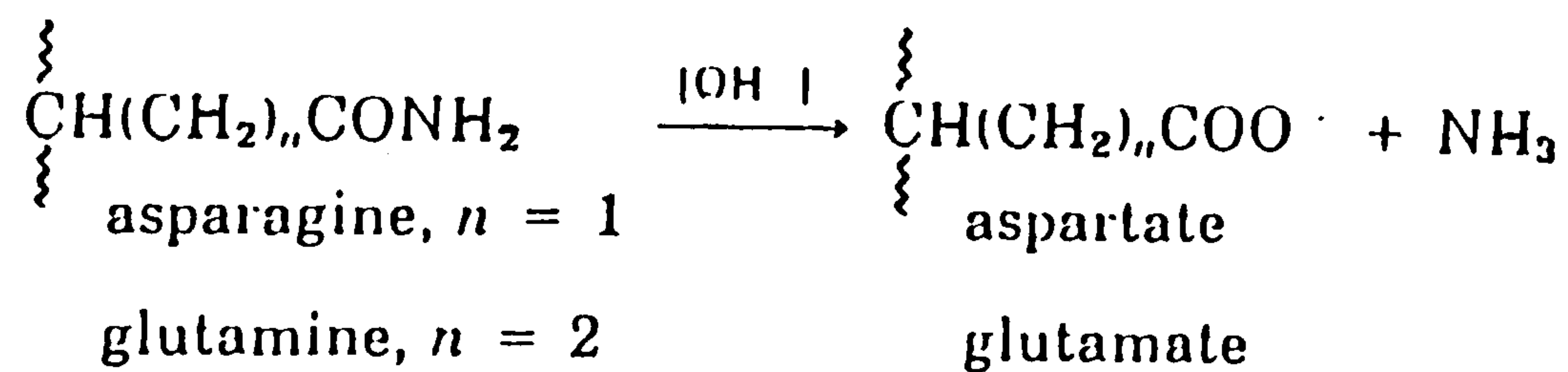


Figure 2.12. The chemical hydrolysis of asparagine and glutamine amino acid residues to aspartate and glutamate respectively; the reaction is particularly favoured by the liming process.

### Pretreatment

Pretreatment swells and softens the hides or ossein prior to denaturation and extraction; peptides and crosslinks are hydrolysed and impurities are eliminated. Alkali and acid pretreatment are the two major procedures used. Alkali pretreatment is generally used for ossein and hides, whereas acid pretreatment is almost always used for pigskins and sometimes for ossein. Alkali pretreatment produces what are termed Type B gelatins, whereas acid pretreatment produces Type A gelatins.

The alkali procedure usually involves soaking the raw stock in large vats of calcium hydroxide suspension, at ambient temperature, for periods of time ranging from several weeks to several months (liming). In the early stages of liming, amidolysis of the asparagine and glutamine amino acid residues occurs, converting them to aspartate and glutamate respectively (Fig. 2.12); this has the effect of reducing the isoionic point (pI) of the derived gelatin. Underliming and overliming reduce the gelatin yield and affect physical properties, but obtaining the precise conditions required for maximum yield remains somewhat of an art. After liming, the stock is neutralised with dilute mineral acid before the extraction process is commenced.

10-15% sodium hydroxide containing sodium sulphate is sometimes used as an alternative to liming for ossein. This treatment requires between one and three days and transforms the ossein into a fibrous mass which is neutralised, washed and solubilised by heat to produce a single grade of high quality gelatin.

Acid pretreatment differs from liming in both duration and amidolysis. Typically, the process involves soaking pigskin in dilute mineral acid for up to 48 hours. This short time period reflects the number of acid-labile peptide bonds and crosslinks in collagen. Under acid conditions amidolysis proceeds extremely slowly, so that few, if any, amide groups are converted to carboxylates, resulting in higher isoionic points than for alkali pretreated gelatins. Following the acid soak, the stock is washed with fresh water and adjusted to the correct extraction pH.

### Extraction

After pretreatment and washing, the collagen is extracted with hot water, which causes denaturation of the helical conformation, additional hydrolysis, and solubilisation of the gelatin.



The extraction process is carried out in stages of increasing time and temperature (usually 5-10°C rise per stage). After each stage, termed a "cook", the liquor containing between 3-10% gelatin is drained off, fresh water added and the process repeated at a higher temperature. The number of extraction stages is dependent on the company, but can vary from four to as many as ten.

The gelatin "cuts" from different extracts have different physical and chemical properties due to the increasing severity of treatment. The first extracts tend to be high quality gelatins, with high bloom values (gel strengths), whereas later extracts are of a lower quality.

After extraction, the dilute liquors are rapidly processed above 40°C, to avoid gel formation and thermal and biological degradation; they are clarified by filtration, concentrated up by flash evaporation to 25-35% and deionised using ion exchange columns. The extracts are then dried to 9-12% moisture content by crash cooling to a firm gel and blowing with warm air. Finally, the gelatin is ground into granules, blended with other cuts to produce specific physical properties, and packed. Fig. 2.13 shows schematically the procedures adopted in gelatin manufacture. For a more detailed discussion on gelatin manufacture, see Veis (1964); Johns and Courts (1977); Hinterwaldner

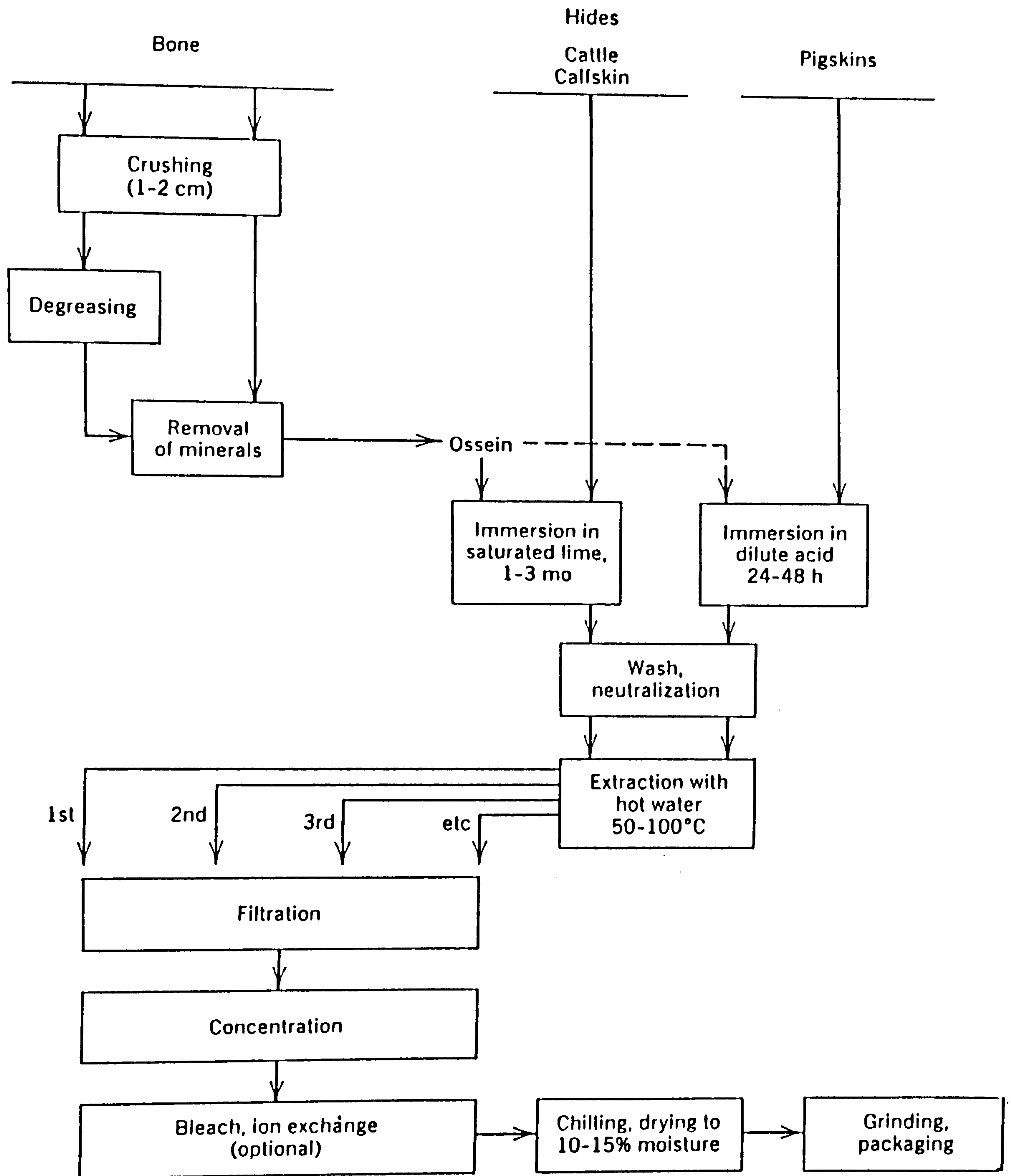
Gelatin Manufacture

Figure 2.13. The processes involved in gelatin manufacture.  
(From Rose, 1986).

(1977); Rose (1986). The properties of gelatins obtained from different extracts and pretreatments will be discussed more fully in Chapter 4.

## 2.5. PROPERTIES OF GELATINS

### 2.5.1. Composition of Constituent Chains

The molecular composition of different gelatins can vary considerably, depending on the species and tissues from which they originate and the methods of pretreatment and extraction.

Generally speaking, mammalian gelatins are remarkably similar in amino acid composition, even though the species from which they are obtained can be extremely diverse. As with the precursor collagens, a large proportion of the amino acid residues are glycine ( $\sim 1$  in 3) while alanine and the imino acids contribute approximately another third.

The remaining amino acids found in gelatin are those which are commonly found in other proteins although there is a noticeable deficiency in tyrosine, methionine, tryptophan, cystine and histidine. Fish gelatins possess a wider range of composition than mammalian gelatins. This is probably a result of the



longer period of evolution to which fish have been subjected, during which time changing environmental conditions, particularly temperature, must have played a role in dictating the various collagen amino acid sequences. The hydroxyproline content in fish gelatins is lowest for cold-water fish (cod and halibut) but higher for hot water fish (lungfish) and it seems likely that this reflects the increased thermal stability of collagen helical structures with increasing hydroxyproline content (See Section 2.3.2.) Invertebrate gelatins similarly show a greater range of amino acid composition, with particularly large variations in the imino acid contents of different species. (For a more detailed account of collagen and gelatin amino acid compositions see Eastoe, 1967).

Species-to-species variation in gelatin composition is, however, of only minor significance when commercial gelatin is considered. This is because, although the study of many different gelatins is of importance for research, most commercial gelatins are obtained from either the pig or the cow. The composition of commercial gelatins will now be discussed further, with reference to the parent collagens and production procedures.

Typical compositions, expressed in terms of specific amino acid residues per 1000 residues, are listed in Table 2.2 for an acid pretreated (Type A) gelatin, a

lime pretreated (type B) gelatin and Type I collagen. It can be seen that the amino acid compositions of the gelatins parallel closely those of the collagen from which the gelatin is derived. However, there are also differences in composition superimposed on this close similarity (Eastoe, 1955). The major difference occurs, as mentioned previously, as a result of the amidolysis of the asparagine and glutamine in the parent collagen (Fig. 2.12). This occurs most readily under alkaline conditions and to a lesser extent under acid conditions, the effect being to alter the isoionic pH (i.e. the pH of a dilute solution which has passed through both cation and anion exchange resins) of the gelatin by increasing the number of free carboxyl groups in the sidechains. The isoionic pH is generally close to the isoelectric pH (Rose, 1986), although the latter can vary depending on the concentration of other solutes in the system. From a knowledge of the amide nitrogen content of a gelatin, it is possible to estimate its isoionic pH, and vice versa, as most samples fall on a smooth curve constructed from a plot of amide nitrogen versus isoionic pH for many different gelatins (Fig. 2.14).

A second, but considerably slower, reaction occurring under alkaline conditions is the conversion of guanidino groups of arginine to amino groups, with the liberation of urea. This process has only a small effect on the overall amino acid composition of

Table 2.2. Amino acid compositions of gelatins and collagen  
(in terms of residues per 1000 residues).

Name	Code	Gelatin		Type-I collagen
		Type-A	Type-B	
alanine	A	112	117	114
arginine	R	49	48	51
asparagine	N	16	0	16
aspartic acid	D	29	46	29
cysteine	C		0	
glutamic acid	E	48	72	48
glutamine	Q	25	0	25
glycine	G	330	335	332
histidine	H	4	4.2	4.4
hydroxyproline		91	93	104
hydroxylysine		6.4	4.3	5.4
isoleucine	I	10	11	11
leucine	L	24	24.3	24
lysine	K	27	28	28
methionine	M	3.6	3.9	5.7
phenylalanine	F	14	14	13
proline	P	132	124	115
serine	S	35	33	35
threonine	T	18	18	17
tryptophan	W			
tyrosine	Y	2.6	1.2	4.4
valine	V	26	22	22



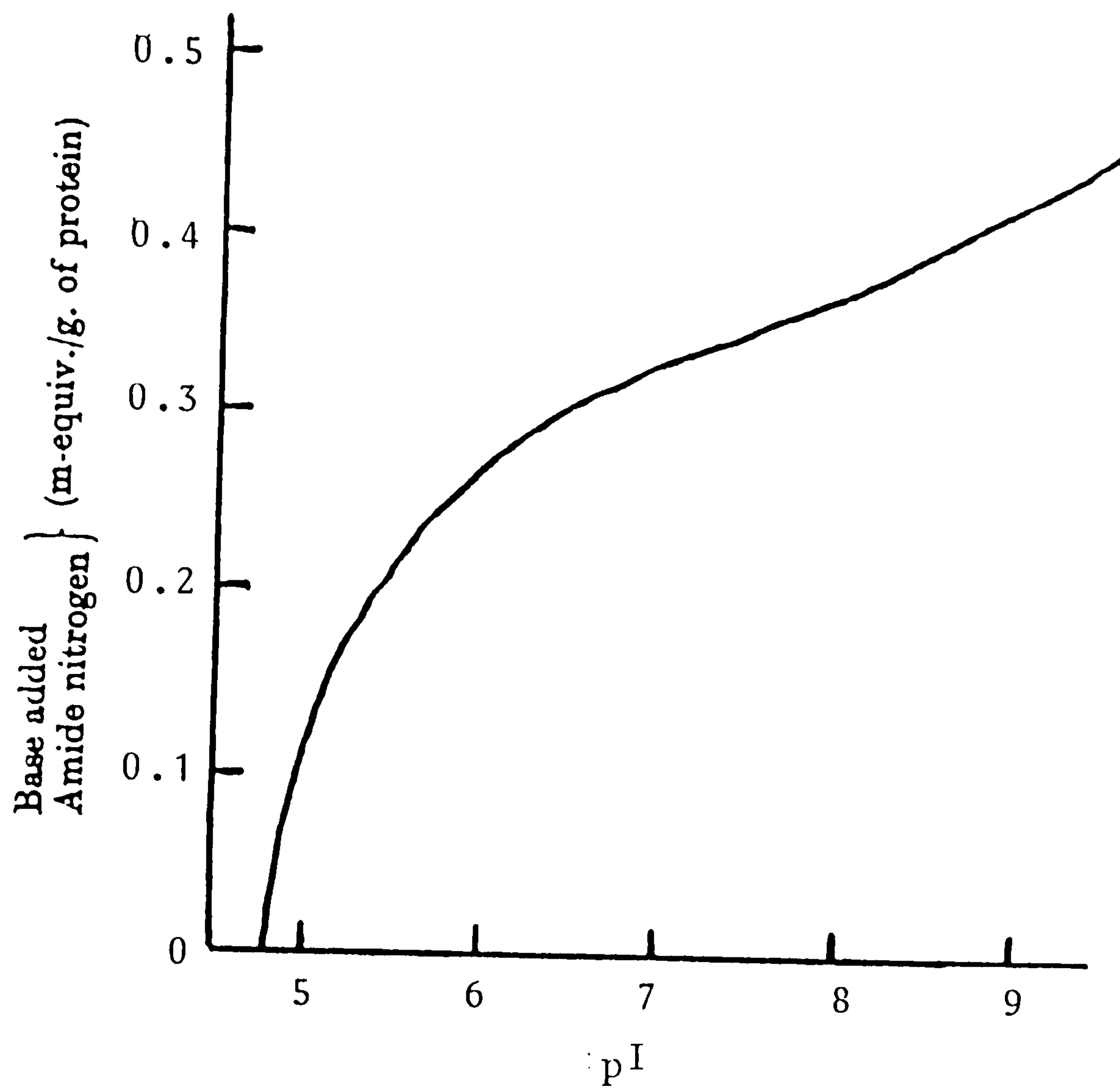


Figure 2.14. Curve showing the relationship between amide nitrogen content and isoionic point (pI) for a range of gelatins (After Eastoe and Leach, 1977).

gelatins and, under typical lime processing conditions, corresponds to conversion of approximately 3% of the arginine residues to ornithine (Eastoe, 1955).

A final difference observed in the amino acid compositions of gelatins compared to their parent collagens is a slight general enrichment in the proportion of the abundant amino acids with a corresponding deficiency in the rarer residues. These differences have been attributed to purification during gelatin manufacture by removal of non-collagenous proteins, and also to the selective removal of a specific part of the gelatin precursor during alkaline pretreatment (Eastoe and Leach, 1977).

Consequently, from the above discussion, it can be seen that the pH behaviour of a gelatin sample is highly dependent on its method of extraction.

Furthermore, the individual molecules of commercial gelatin are heterogeneous with respect to amino acid composition, as polypeptides of equal length, but different amino acid composition, can be produced by cleavage at different positions along the  $\alpha$  chains (Rose, 1986). This heterogeneity is an explanation of the distribution of isoelectric pH observed in isoelectric focussing measurements (Maxey and Palmer, 1976). Further evidence of the heterogeneity of the gelatin chains has come from end group analysis. The

fluorodinitrobenzene method for determination of N-terminal groups has been utilised in a number of investigations (Grassman and Hörmann, 1953; Bowes and Moss, 1953) including a particularly extensive study by Courts (1954, 1959, 1960). The results showed up to eight types of N-terminal groups in various gelatin samples, emphasising the largely unspecific nature of the hydrolytic breakdown of the gelatin precursor.

In addition to the peptide backbone, various unusual molecular structures are thought to occur to some extent in most collagens and gelatins. These unusual features include aldehyde groups, possibly present as a result of the degradation of the aldehyde-based crosslinks mentioned in Chapter 1, 'esterlike' bonds,  $\gamma$ -glutamyl linkages and carbohydrates (see Harding, 1965 and Gallop *et al.*, 1967).

Other important factors which can influence the properties of gelatin preparations are the impurities, most notable of which are inorganic substances (either counterions or free salts) and other proteins and mucosubstances present as a result of residual material extracted from the animal tissues along with the gelatin.



### 2.5.2. Molecular Weights and Sizes of Gelatins

In addition to the chemical constitution of gelatin, it is also necessary to consider the molecular size and weight averages of the chains and indeed, possibly even of greater significance, the molecular weight distribution profiles. This is because it is the size of gelatin chains which determine, to a large extent, the rheological properties of gelatin solutions.

The molecular weight of a gelatin is thought to be close to 90,000. However, commercial gelatins with molecular weights closer to 200,000 have been observed using light scattering studies (Gouinlock *et al.*, 1955) and sedimentation experiments (Williams *et al.*, 1954). Stainsby *et al.*, (1961) have confirmed the occurrence of such high molecular weights, reporting values as high as 1 million for the higher fractions of some gelatins, and it is now well established that these gelatins are molecularly disperse (i.e. the high molecular weights are not a result of aggregation of chains in solution (Stainsby, 1977a)). The high molecular weight fractions have been shown (Courts and Stainsby, 1958; Stainsby *et al.*, 1961) to be multichain structures, by use of end-group analysis and light scattering to calculate the number of chains per molecule. These multichain structures are thought to originate from retention of some covalent crosslinks

from the collagen molecule and not as any consequence of the procedure used in gelatin manufacture (Stainsby, 1977a). More recently, molecular weight distribution profiles of gelatins have been obtained using gel permeation chromatography and gel electrophoresis. Typical commercial gelatins show polydisperse molecular weight distributions which can be resolved into numerous peaks, the pre-treatment and extraction processes controlling, to a certain extent, the specific molecular weights obtained (See also Chapter 4).

The actual size and shape of gelatin molecules in solution have been studied, but interpretation of the results has proved to be difficult. There is no single relationship between intrinsic viscosity and molecular weight for all gelatins in a given solvent, with different methods of manufacture producing differing values for the Mark-Houwink parameters  $K$  and  $a$  (see Section 3.2.2.). Light scattering measurements give information about the shape of molecules, providing the wavelength of the light is sufficiently small compared to the particle size, but such studies on gelatin have often been inconclusive due to complications from impurities (Stainsby, 1956; Tabor, 1962) and aggregation. Results from the work of Boedtker and Doty (1954) indicated that gelatin molecules were present in solution as straight-chain molecules in a typically "random-coil" type conformation. (A larger degree of branching would



cause the molecules to appear more compact). It has also been suggested that disordered gelatin molecules exist in an extended coil conformation as a result of the large number of rigid imino acid residues in the primary sequence (Silver and Trelstad, 1981).

### 2.5.3. Solution Properties of Gelatins

Addition of cold water to a gelatin sample causes the sample to gradually swell, taking up the water. Except in the case of certain highly degraded samples, however, gelatin does not dissolve in cold water. Subsequent heating of the swollen gelatin to temperatures above 40°C causes solubilisation, producing a solution of conformationally-disordered gelatin chains. (40°C is therefore generally regarded as the minimum temperature for molecular characterisation). Certain denaturing solutes such as urea, guanidinium ion, LiBr, KSCN, etc., at sufficiently high concentrations, promote solubility, and under such conditions gelatin will dissolve at low temperatures without any swelling occurring (Stainsby, 1977a).

Gelatins are rich in both acidic and basic functional groups, imparting considerable polyampholyte character to gelatin solutions. Thus, depending on pH,



gelatins can display Donnan membrane effects with simple ions of either positive or negative charge and behave as both cation and anion exchangers (Rose, 1986).

Veis (1964) has reviewed the titration behaviour of gelatins, and some of the effects of pH/ionic environment on the properties of gelatin solutions will now be further considered.

Gelatins, except in the case of certain chemically modified gelatins, are soluble over the whole pH range, producing clear solutions which are usually tinted slightly yellow. At pH values close to their isoionic point the solutions can become slightly hazy as a result of aggregates caused by electrostatic forces, although in most commercial preparations the salts present cause a high enough ionic strength to prevent this. Buffers and neutral salts do not generally decrease the solubility of gelatin with polyhydric compounds similarly not causing gelatin to precipitate out of solution. Alcohols and ketonic compounds, on the other hand, cause precipitation when they are present in excess, as can other charged polymers if the sign of the charge is opposite to the overall charge on the gelatin; in this way mutual precipitation by different gelatins can occur. The use of alcohols in molecular weight fractionation of gelatin, via the formation of coacervates, has been

widely used (Stainsby, 1977a), while the formation of gelatin coacervates also has important industrial uses in microencapsulation, photographic emulsions and coatings applications.

The viscosity of gelatin solutions, particularly at low concentration, can show an appreciable dependence on the pH and nature of other solutes in the system. As in all polyelectrolytes, the coil dimensions of the gelatin chains and hence solution viscosities, are dependent on two main factors. Firstly, there is the effect of the net charge on the polymer, be it positive or negative, causing coil expansion via intramolecular electrostatic repulsion and secondly, the effect of simple electrolyte concentration screening the repulsions and consequently allowing the coils to contract. Addition of acid or alkali to gelatin solutions can result in complicated interplay between these two effects as illustrated in Figs. 2.15a and 2.15b which show the variation in reduced viscosity with pH for an alkali-pretreated gelatin and an acid-processed gelatin respectively.

For both gelatins the reduced viscosity is minimum at the isoelectric point and rises to maximum values at approximately pH3 and pH10.5. At the isoelectric point the net charge on the gelatin molecules is zero and so intramolecular electrostatic repulsion is minimised, resulting in the smallest coil dimensions.

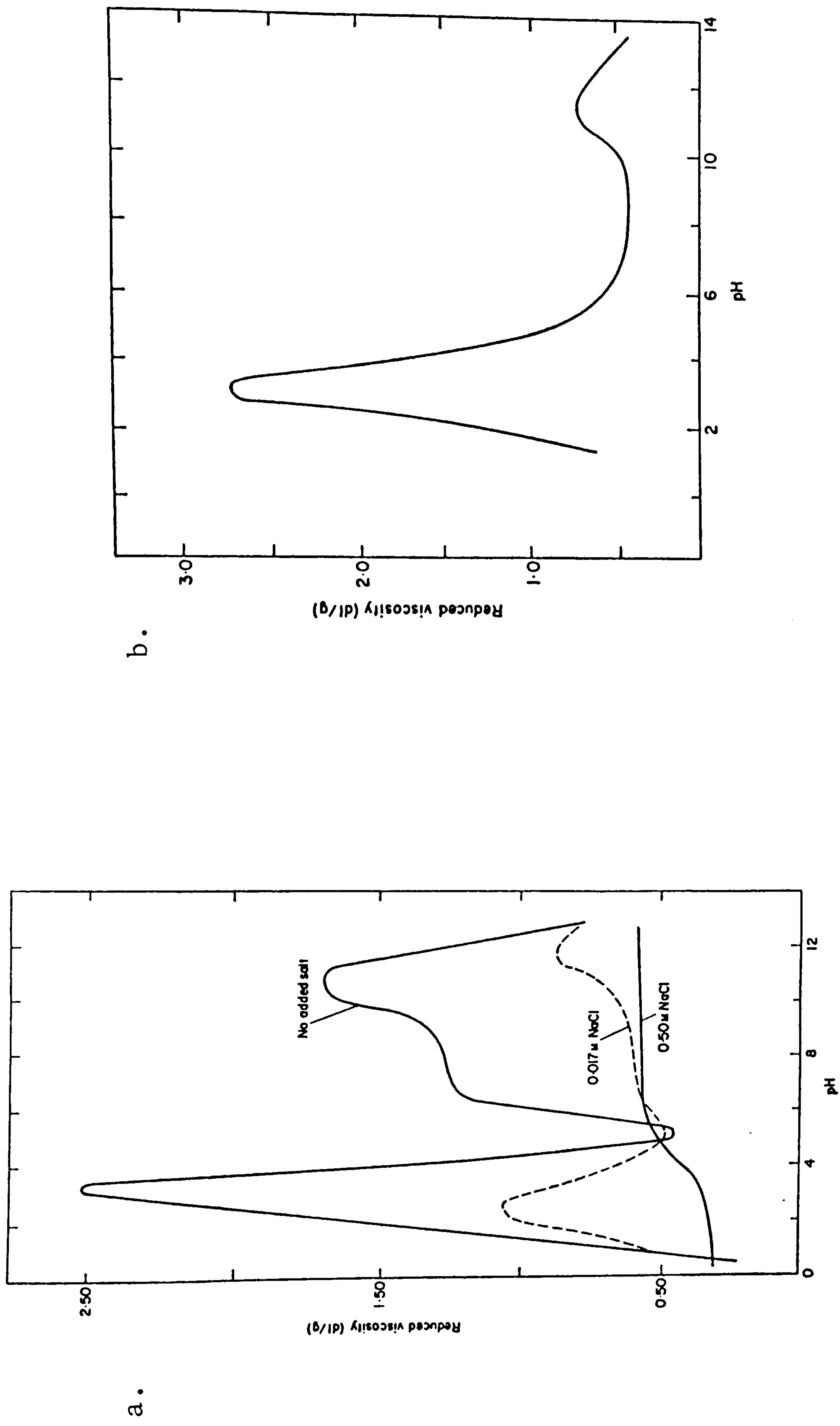


Figure 2.15. The effect of pH on the reduced viscosity of gelatin solutions (0.20%) at 38°C.  
 a) A low pI gelatin in the presence of various amounts of added salt.  
 b) A deionised acid-process pigskin gelatin.  
 (From Stainsby, 1977a).



An increase or decrease in pH causes an increase in charge density on the gelatin molecules which consequently expand producing an increase in viscosity. However, an increase or decrease in pH also causes an increase in ionic strength of the solution, with subsequent shielding of the expansion effects. Under very strong acidic or basic conditions, where ionisation of the gelatin is virtually complete, the shielding effects become dominant causing the gelatin coils to contract and the observed decrease in reduced viscosity.

The addition of neutral salts (see Fig. 2.15a) also increases the ionic strength of the solution reducing the dependence of reduced viscosity on pH. For these reasons intrinsic viscosity measurements on gelatins should be carried out at constant pH in the presence of sufficient electrolyte to produce a higher, constant, ionic strength.

The choice of pH when working with gelatin solutions is also important from the point of view of degradation reactions. All gelatins will degrade due to hydrolysis of covalent crosslinks and peptide bonds, the rate at which this occurs increasing substantially with increasing temperature and deviation from neutrality. Furthermore, bacterial contaminants in gelatin can produce enzymes which also cause degradation; these processes are again

highly dependent on temperature and pH. For the above reasons all the solutions of gelatin used throughout the work reported in this thesis were prepared in 0.1M sodium chloride at pH7 with sodium azide present as a bacteriocide.

In common with all proteins, gelatins exhibit optical rotation due to the chirality of their amino acids. However, the specific rotation of a warm gelatin solution is more laevorotatory than would be expected if the rotation was simply a result of the amino acids linked in an entirely random configuration (Harrington, 1958). It was suggested that the higher value of the optical rotation was a consequence of certain regions of the polypeptide chain being locked in a polyproline II-like conformation (i.e. even at high temperature some parts of the chains were existing in a conformation similar to that of native collagen). Such behaviour is not at all improbable as the Gly-X-Y triplets, in which X and Y are proline or hydroxyproline, are further restricted in terms of free rotation compared to triplets containing non-imino acids, due to the rigid nature of the imino group. LiBr and KSCN, at high enough concentrations, can collapse the regions of polyproline II-like structure, and when such salts are present in gelatin solutions the excess optical rotation disappears. The explanation is not as simple as this, however, as it has been shown that the loss of laevorotation under such conditions is greater than expected (Harrington, 1958).



In contrast to the situation at higher temperatures, where the optical rotation of a gelatin solution is independent of time, at lower temperatures (below the gel melting temperature), there are marked changes in optical rotation with time. On cooling a gelatin solution to a specific temperature, there is first a rapid increase in laevorotation, the rate of increase gradually decreasing with time; the actual value of the rotation, however, never reaches a plateau and seems to continue increasing indefinitely. The large increase in the magnitude of the optical rotation is attributed to some partial regeneration of a collagen-like structure, with the value of optical rotation at any time providing a measure of the extent of conformational ordering. Consequently, the technique of optical rotation has frequently been utilised in studies of gelatin renaturation; this will be discussed further in Chapter 6.

#### 2.5.4. Properties of Gelatin Gels

Providing the gelatin concentration is high enough (usually greater than 0.5% for most commercial gelatins), adoption of the triple-helix structure is accompanied by formation of a clear gel, the rheological properties of which are dependent on a



number of factors (e.g. temperature, time, gelatin concentration, added solutes). The properties of gelatin gels have been the subject of numerous investigations throughout the years, as a result of their wide application within the food and photographic industries, and there are many excellent reviews which deal with the subject (Saunders and Ward, 1958; Ward, 1959; Idson and Braswell, 1957, 1960; Veis, 1964; Stainsby, 1977b; Ledward, 1986). One property of paramount importance when considering gelatin gels is their thermoreversibility and, more specifically, the temperature range over which they melt. The fact that gelatin gels melt just below body temperature gives unique organoleptic properties and "melt in the mouth" characteristics compared to other gelling hydrocolloids such as polysaccharides, which generally have much higher melting temperatures (usually greater than 40°C). A further advantage of gelatin gels over many polysaccharide gels (carrageenan, alginate, gellan) is that they are not dependent on the presence of salts to cause gelation. They are also stable over a wide pH range at the usual concentrations used. Figs. 2.16b and 2.16a illustrate the effect of pH on the melting temperature of an acid pretreated and alkali pretreated gelatin respectively. Only at gelatin concentrations close to the threshold of gelation does pH have any significant effect on the melting point of the gel. Furthermore, even these pH effects at low concentrations can be eliminated by the addition of

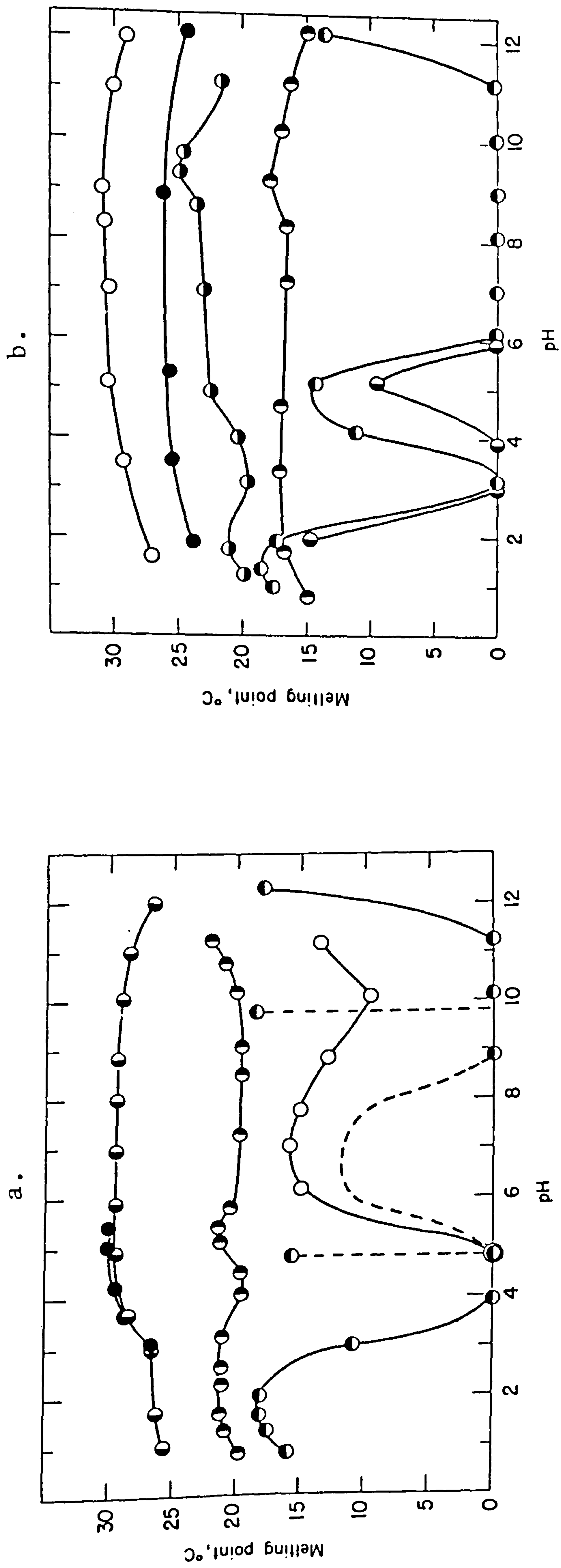


Figure 2.16. The effect of pH on the melting points of gelatin gels at various concentrations and in the presence of salt.

a) Alkali process gelatin. Deionised: ○, 5% calf skin; ●, 5% ossein; ○, 0.57% calf skin (dotted curve indicates that weak gels formed at pH 6, 7, and 8 but that gel strengths were not measured; vertical dashed lines show the effect of 0.1M sodium chloride); ○, 0.7% calf skin. Non-deionised: ○, 0.7% ossein.

b) Acid process pig skin gelatin. Deionised: ○, 5%; ●, 1.2%; ○, 0.7%; ○, 0.6%; ○, 0.6% with 0.1M sodium chloride added. Non-deionised: ●, 0.73%.

(From Veis, 1964)



sufficient salt, and this is another reason for using 0.1M NaCl as solvent, wherever possible throughout this work.

At a macromolecular level, gelatin gels can be viewed as an intermolecular, three-dimensional network, formed by partial regeneration of the gelatin molecules into ordered junction zones linked by residual disordered chain regions. Naturally, the concentration of the gelatin solution plays an important role in determining the properties of the gel, in particular the gel strength. The rigidity,  $G$ , has frequently been used as a measure of the elastic properties of gels, but great care must be taken when comparing rigidity values of gels of different concentrations to ensure that they have been subjected to exactly the same time and temperature regime, as these factors too can influence the rigidity values obtained (see Chapters 5 and 6). Early studies suggested that rigidity was proportional to the square of concentration (Leich, 1904; Poole, 1925; Sheppard and Sweet, 1921; Kinkel and Sauer, 1925), but later work by Ferry and Eldridge (1949) indicated a deviation from the square-law at lower concentrations. Saunders and Ward (1954) found no linear relationship between rigidity and the square of concentration, and they concluded that there was probably no general relationship between concentration and rigidity that could be applied to all gelatins (Ward and Saunders,



1958). More recently, however, a general relationship between gel modulus and concentration has been developed from cascade theory (Clark and Ross-Murphy, 1987) and verified experimentally for several biopolymer systems including gelatin (Clark and Ross-Murphy, 1985; Clark *et al.*, 1983).

Industrially, measurements of gel strength are made using a gelometer, which determines the force in grams (called grams Bloom) required for a standard diameter plunger to deform the surface of the gel by 4mm (Rose, 1986). The Bloom values quoted are obtained using a 6.67% w/v solution (uncorrected for moisture and ash) chilled in a standard container at 10°C for 16-18 hours.

As in all gel systems, gelation will not occur at all below a certain critical concentration. At these low concentrations, cooling a gelatin solution still causes the characteristic changes in physical properties associated with conformation change (e.g. large increases in laevorotation, increases in viscosity), even though the solution does not set to gel. Consequently, studies of the dilute solution behaviour of gelatin have frequently been used to investigate the molecular processes involved in gelation without the added complications that network formation can cause. The different approaches and mechanisms proposed for gel formation will be discussed more fully in Chapters 6 and 7, in the light of experimental work presented in this thesis.

## CHAPTER 3: PHYSICAL TECHNIQUES

### 3.1. GENERAL INTRODUCTION

Current fundamental understanding of the structure and mechanical properties of solutions and gels formed by biopolymers has been largely obtained through the application, to these systems, of various physical techniques. The aim of this chapter is to illustrate the principles of the techniques utilised in this thesis, and more specifically, to illuminate the manner in which they have been employed for the study of gelatin gelation.

As outlined in Chapters 1 and 2, the physical properties of a gelatin solution can be substantially altered by the random coil  $\rightarrow$  triple helix conformation change. Such conformation changes are often observed in biopolymers and their study is thus of primary importance in the determination of physical and gelling properties. Thermally-induced order-disorder transitions, of which gelatin is a notable example, provide direct evidence of conformational order and may be monitored by discontinuities in the temperature course of physical properties such as rheology, optical activity and heat capacity. The principles behind these three techniques will now be discussed in more detail with particular emphasis on their application to biopolymers.

## 3.2. RHEOLOGICAL TECHNIQUES

### 3.2.1. Rheology of Gels and Solutions

The application of rheological techniques to gelling systems can provide valuable information about the macromolecular and macroscopic properties (e.g. overall molecular shape, aggregation and intermolecular network formation) of the system under study. Rheological studies of solutions or gels fall into one of two broad categories:

- 1) Small-deformation measurements, in which the structure is retained.
- 2) Large-deformation studies, in which breakdown of structure is measured.

While both techniques are extremely useful, the former has the advantage of being non-destructive, and is therefore ideal for monitoring systems under circumstances where numerous measurements must be carried out on one sample under different conditions (e.g. at different temperatures when melting a gel).



The fundamental operation in all rheological testing is to apply a force to the sample under investigation and measure its deformation, or alternatively to apply a deformation and measure the resistance. Application of such a regime yields two idealized extremes: perfect (elastic) solids and perfect (Newtonian) liquids. The former obey **Hooke's law**, where stress (force per unit area) is directly proportional to strain (fractional deformation) the ratio of stress : strain giving a measure of the elasticity of the material. In the latter case the resistance of a Newtonian liquid to imposed movement is directly proportional to the rate of strain, but independent of the strain itself; the ratio of stress : rate of strain is the viscosity of the liquid ( $\eta$ ). The fundamental rheological parameters and their standard symbols for longitudinal and lateral deformation are summarised in Table 3.1.

Biopolymers possess properties intermediate between the two extremes of idealized behaviour (Gibbs *et al.*, 1968; Morris and Ross-Murphy, 1981), that is, they display both liquid-like and solid-like character and are thus referred to as being viscoelastic. The two modes of response to an applied deformation can be quantitatively resolved by the powerful technique of mechanical spectroscopy (Ferry, 1980). The sample under test is subjected to a sinusoidal deformation of low amplitude (maximum strain typically  $<10\%$ ) and frequency,  $\omega$ ; the resistance to distortion is then

Table 3.1. The various Rheological Parameters.

PARAMETER (units)	LONGITUDINAL DEFORMATION (compression or extension)	LATERAL DEFORMATION (shear)
STRESS (Pa; 1 Pa = 10 dyne cm <sup>-2</sup> )	$\sigma$	$\tau$
STRAIN (dimensionless)	$\epsilon$	$\gamma$
STRESS/STRAIN (Pa)	E	G
RATE OF STRAIN (s <sup>-1</sup> )	$\dot{\epsilon}$	$\dot{\gamma}$
STRESS/RATE OF STRAIN (Pa s)	$\lambda$	$\eta$
(1 Pa s = 10 Poise; 1 mPa s = cP)		

measured. For a perfectly elastic solid the stress generated in resisting the applied deformation is exactly in-phase with the imposed strain, whereas for Newtonian liquids the resistance to flow (stress) is maximum at the midpoint of the oscillatory cycle, corresponding to zero strain but maximum rate of strain; in this case, stress and strain are exactly out-of-phase. (See Fig. 3.1). For a viscoelastic material such as a biopolymer solution under oscillatory shear, resolution of the resultant stress into its in-phase and out-of-phase components is possible, providing a measure of the solid-like and liquid-like character of the material. This is naturally of great interest when monitoring a change from solution to gel or vice versa.

The ratio of in-phase stress: applied strain is the elastic modulus,  $G'$  (also known as the storage modulus) while the corresponding ratio of out-of-phase stress : strain is the viscous modulus,  $G''$  (also known as the loss modulus). The overall response of the sample to the applied deformation may be characterised by the complex modulus,  $G^*$ .

$$G^* = (G'^2 + G''^2)^{\frac{1}{2}} \quad (\text{Equation 3.1.})$$



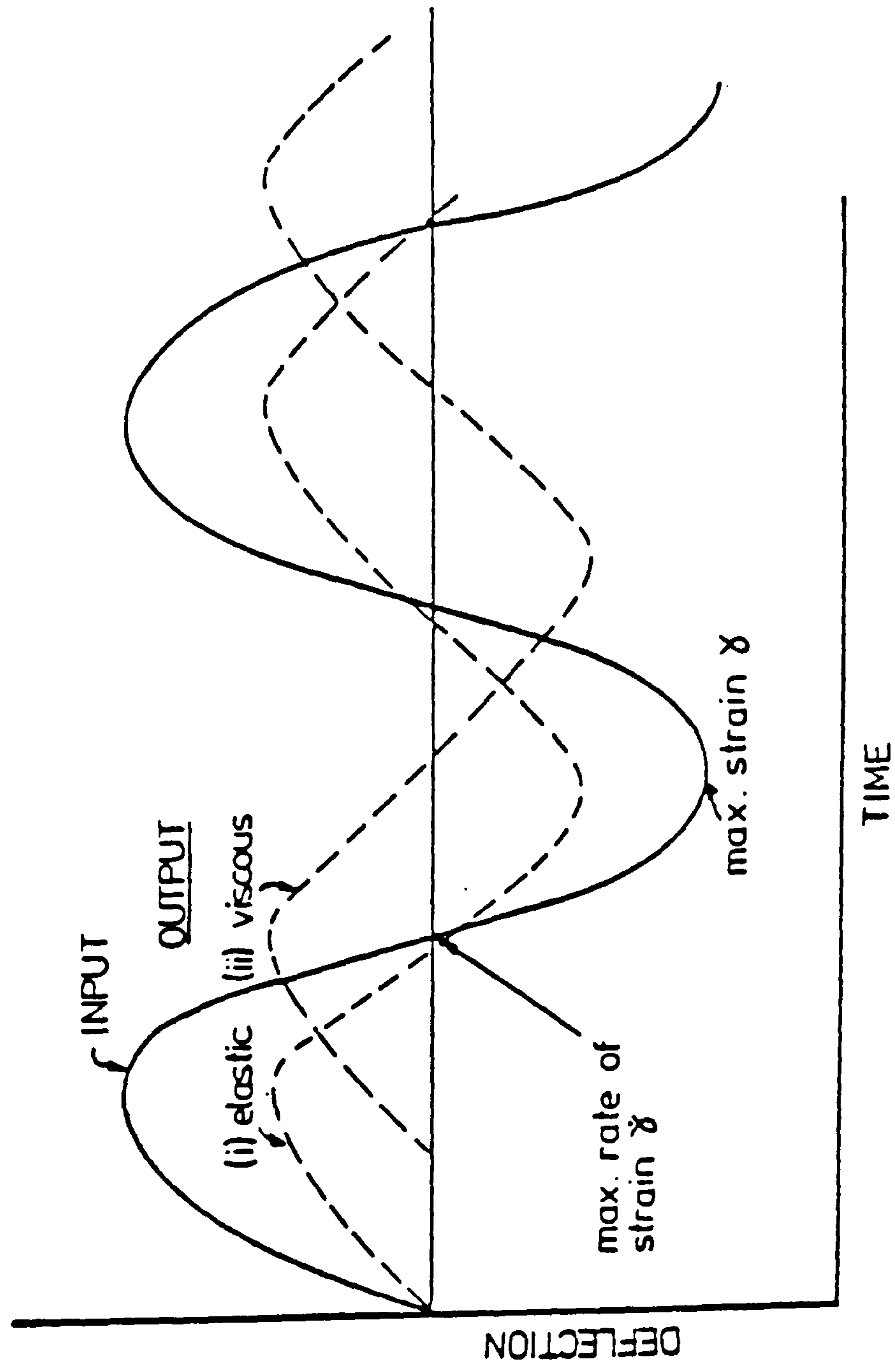


Figure 3.1 Oscillatory measurements illustrating the response of a sample to a sinusoidal deformation (i) a perfectly elastic solid and (ii) a perfectly viscous liquid.

Frequency of oscillation ( $\omega$ ) can be considered as the oscillatory analogue of shear-rate and hence according to table 3.1 it is possible to define a dynamic viscosity ( $\eta^*$ ).

$$\eta^* = \frac{G^*}{\omega} = \frac{(G'^2 + G''^2)^{\frac{1}{2}}}{\omega} \quad (\text{Equation 3.2.})$$

In the technique of Mechanical Spectroscopy, these parameters are measured as a function of frequency of oscillation. For permanent networks (e.g. true polymer gels) samples show properties similar to those of elastic solids:  $G'$  predominates over  $G''$  at all frequencies with neither showing any appreciable frequency-dependence.  $\eta^*$  decreases steeply with increasing frequency, with the slope of a plot of  $\log \eta^*$  versus  $\log \omega$  approaching the theoretical maximum value of -1, as expected from Equation 3.2. (See Fig. 3.2a).

Dilute hydrocolloid solutions, in which polymer chains are free to move individually, are characterised by a mechanical spectrum such as shown in Fig. 3.2b. There is little variation of dynamic viscosity with frequency, particularly at the low frequency end of the spectrum, whilst  $G''$  is substantially higher than  $G'$  (i.e. essentially Newtonian behaviour). At very high frequencies, however,  $G'$  approaches  $G''$  due to storage of energy by contortion of the chains into strained conformations (Graessley, 1974; Ross-Murphy, 1984).

The physical size and extended nature of polymer coils in solution means that, even at relatively low concentrations, the chains can become entangled with one another. Indeed, in large deformation measurements in which the viscosity can be measured as a function of polymer concentration ( $c$ ), a sudden change in the slope of a plot of  $\log \eta$  against  $\log c$  is indicative of the onset of coil overlap (Fig. 3.3) (Morris, 1983); the concentration at which this occurs is known as the critical concentration ( $c^*$ ).

Mechanical spectroscopy on polymer solutions with polymer concentrations greater than  $c^*$  yield a third general type of spectrum (Fig. 3.2c) for so-called 'concentrated solutions'. Here there is a transition from typical liquid-like behaviour ( $G'' > G'$ ) at low frequency, where chains can become untangled in the period of one oscillation, to a more solid-like response ( $G'' < G'$ ) at higher frequencies as a result of insufficient time within the period of one oscillation for disentanglement, the solution thus effectively behaving as a gel. In particular, the mechanical spectra of 'concentrated solutions' have an  $\eta^*$  which is independent of frequency at low frequencies but decreases steeply on reaching higher values of  $\omega$ .



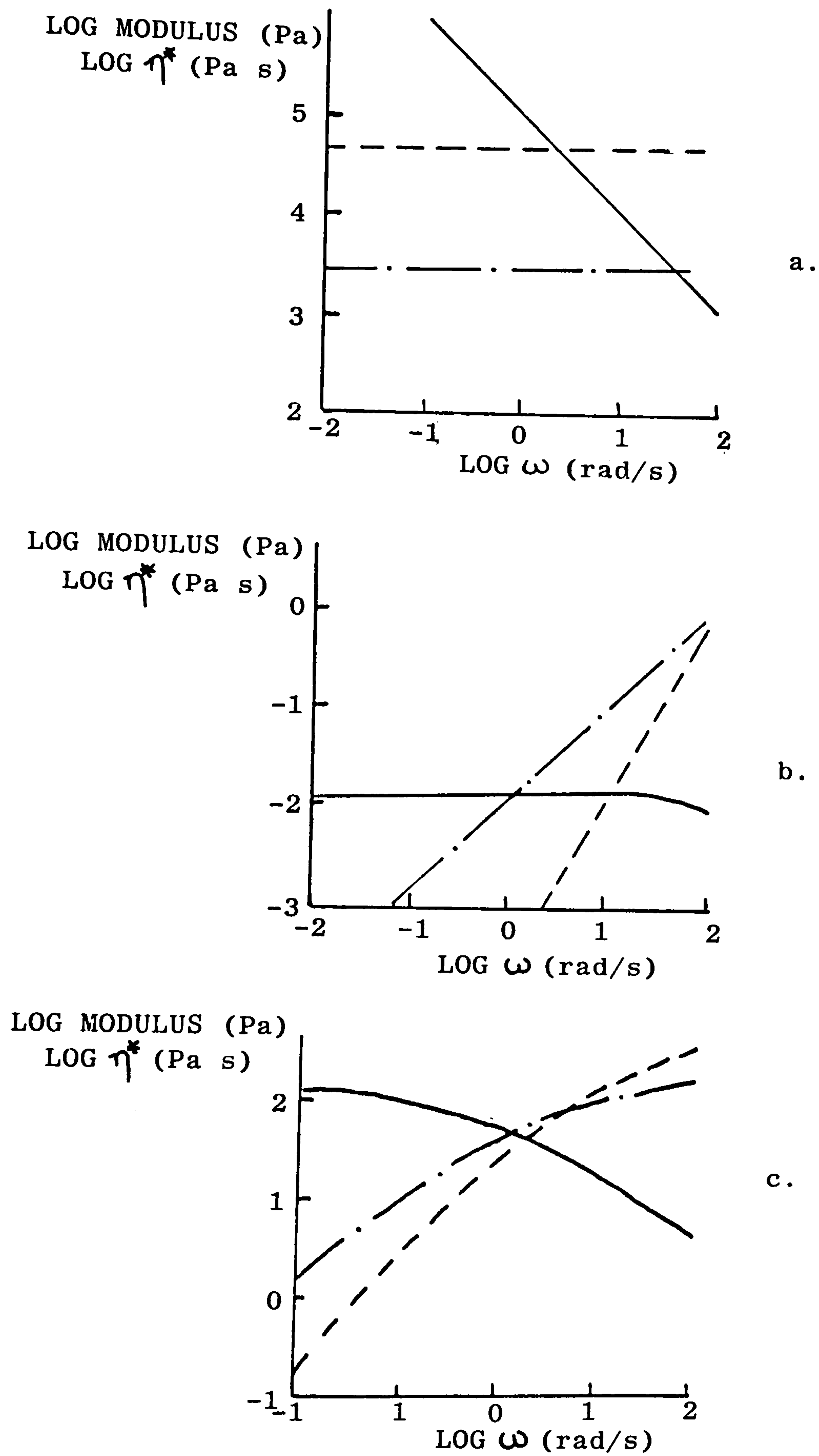


Figure 3.2. Typical mechanical spectra of biopolymer solutions and gels. Solid-like and liquid-like behaviour are characterised by the storage (---) and loss (— · —) moduli  $G'$  and  $G''$  respectively, and the overall response to applied deformation can be represented by the dynamic viscosity  $\eta^*$  (—); a) typical of a strong gel; b) typical of a dilute solution and c) typical of a concentrated solution.

Large deformation measurements on such 'concentrated solutions' show a shear rate ( $\dot{\gamma}$ ) dependence of viscosity. At low shear rates the viscosity is constant but it begins to decrease as the shear rate increases. For this reason, zero shear viscosity ( $\eta_0$ ) is often quoted. This shear rate dependence of viscosity is again a result of polymer entanglement. At low shear rates the polymer chains have enough time to re-entangle with new partners, leading to an equilibrium situation and a constant value of  $\eta$  with respect to shear rate. At higher shear rates, however, there is insufficient time for full re-entanglement, and consequently the viscosity drops; this is known as shear thinning. For many polymer solutions, it has been noted that the frequency dependence of  $\eta^*$  and the shear rate dependence of  $\eta$  are closely superimposable when equivalent values of frequency and shear rate are compared (Cox and Merz, 1958; Morris and Ross-Murphy, 1981). This relationship was originally empirical but is now explained by modern network theory.

### 3.2.2. Hydrodynamic Measurements in Solution

The critical concentration at which coil overlap for a polymer occurs ( $c^*$ ) is, of course, also dependent on the size and shape of the polymer molecules under investigation (i.e. the volume occupied by each molecule in isolation). Experimentally, viscosity measurements at concentrations below  $c^*$  can be used to

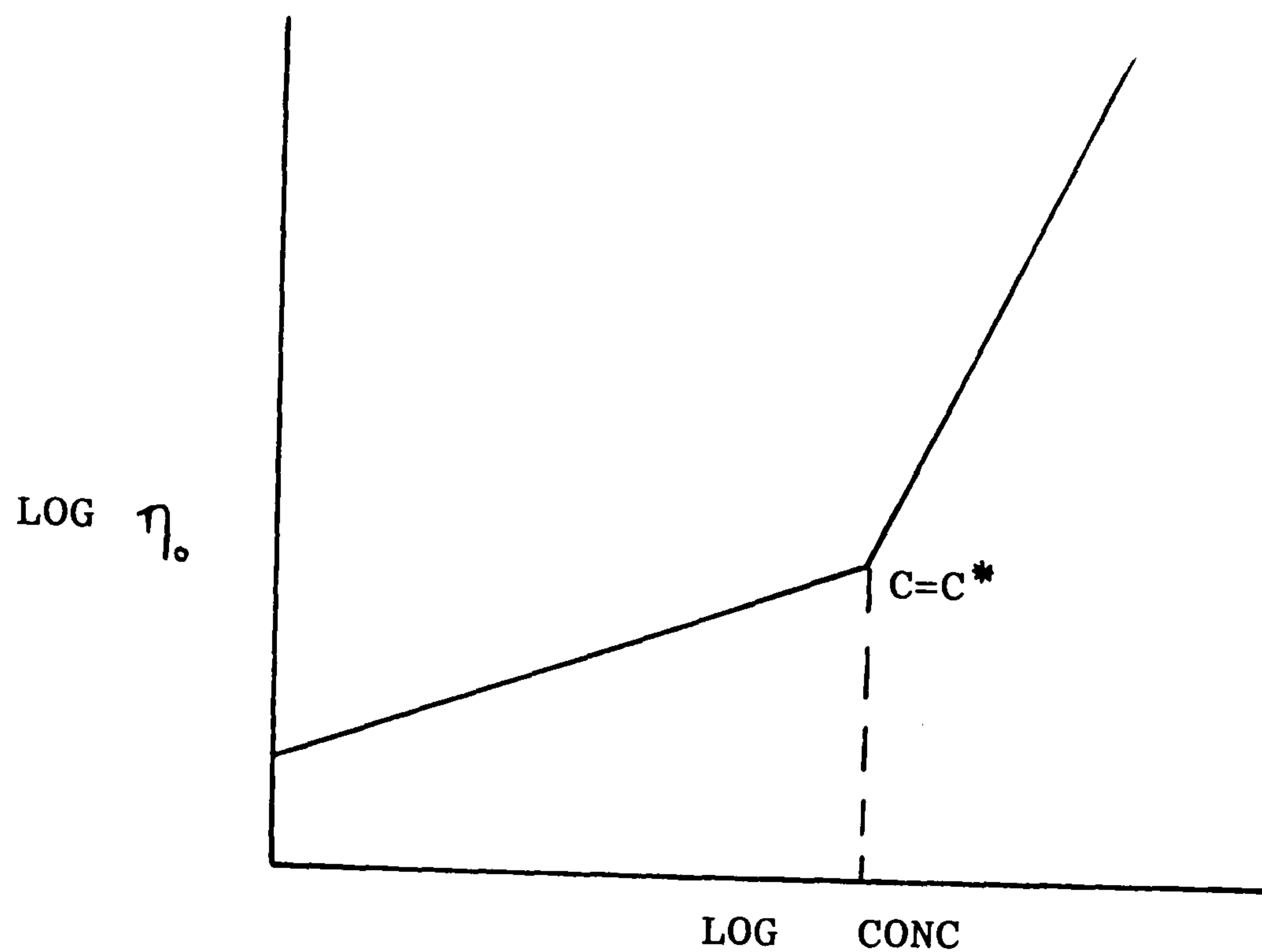


Figure 3.3. The concentration dependence of 'zero shear' viscosity for a typical random coil polymer.  $c^*$  is the concentration at which coil overlap is presumed to begin.

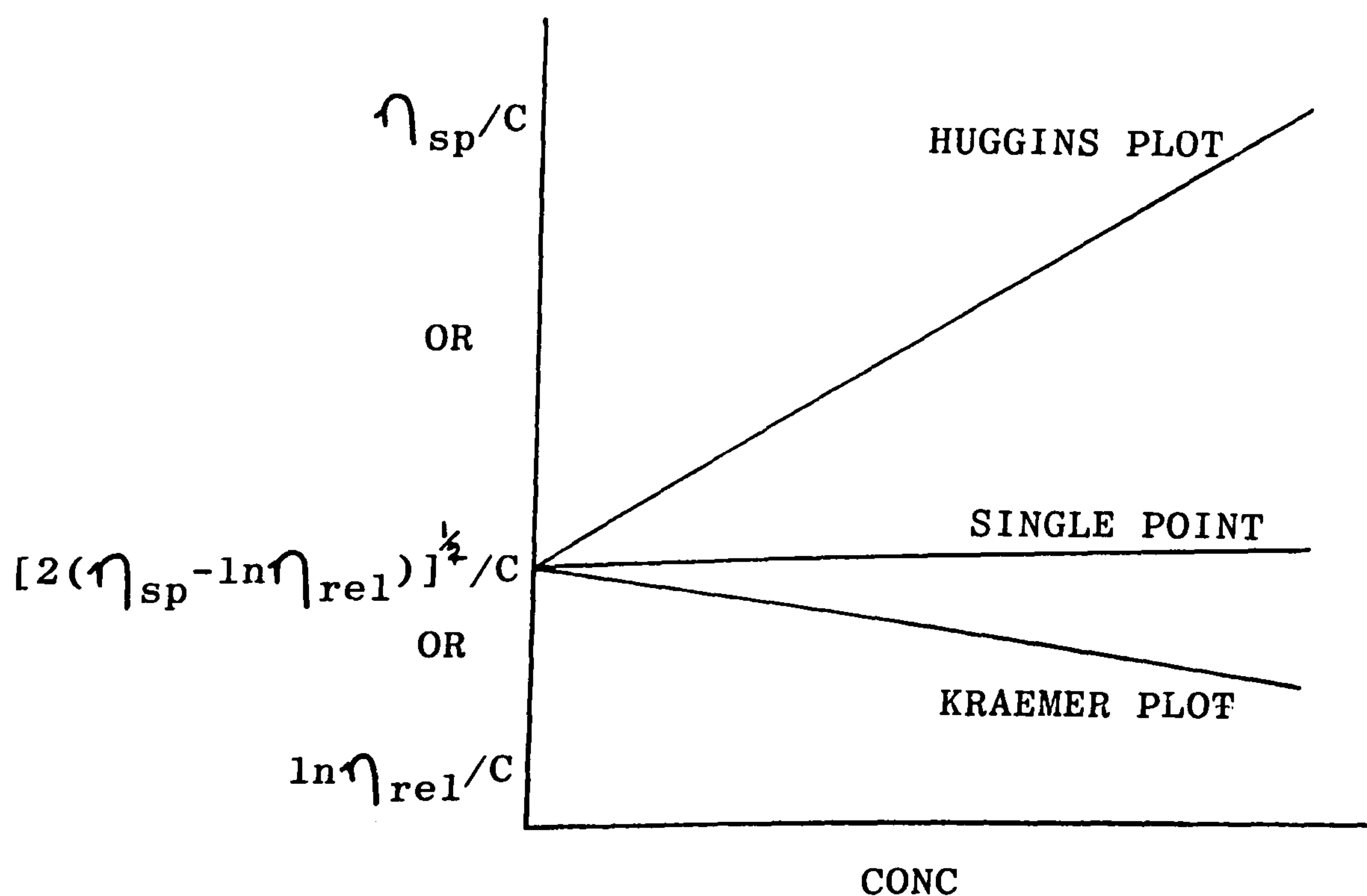


Figure 3.4. The three extrapolations to zero concentration which can be used in the determination of intrinsic viscosity.



characterise the 'hydrodynamic volume' of a polymer coil in terms of its intrinsic viscosity. This is performed by measuring the viscosity of the polymer solution relative to that of the solvent ( $\eta_{rel}$ ).

$$\eta_{rel} = \frac{\eta_{solution}}{\eta_{solvent}} \quad (\text{Equation 3.3})$$

The specific viscosity ( $\eta_{sp}$ ) which is a measure of the relative increase in viscosity due to the dissolved solute is then given by

$$\eta_{sp} = \frac{\eta_{solution} - \eta_{solvent}}{\eta_{solvent}} = \eta_{rel} - 1$$

(Equation 3.4)

Dividing  $\eta_{sp}$  by the polymer concentration gives the reduced viscosity ( $\eta_{red}$ )

$$\eta_{red} = \eta_{sp}/c \quad (\text{Equation 3.5})$$

Finally, in the limiting case where the reduced viscosity is extrapolated to infinite dilution the intrinsic viscosity  $[\eta]$  is obtained

$$[\eta] = \lim_{c \rightarrow 0} (\eta_{sp}/c) \quad (\text{Equation 3.6})$$

Even in dilute solutions, before the onset of entanglement, polymer chains can interact indirectly due to their effect on solvent flow. Quantitatively

$$\eta_{sp}/c = [\eta] + k' [\eta]^2 c \quad (\text{Equation 3.7})$$

Thus, an extrapolation of  $\eta_{sp}/c$  against  $c$  to zero concentration (Huggins plot) gives  $[\eta]$ .

An alternative extrapolation (Kraemer plot) is that of  $(\ln \eta_{rel})/c$  against  $c$  as

$$\ln \eta_{rel}/c = [\eta] + k'' [\eta]^2 c \quad (\text{Equation 3.8})$$

The Huggins and Kraemer constants ( $k'$  and  $k''$ ) are related by the simple expression

$$k' = k'' + 0.5 \quad (\text{Equation 3.9})$$

and thus a third extrapolation may also be utilised in determination of intrinsic viscosity (See Fig. 3.4)

$$[\eta] = [2(\eta_{sp} - \ln \eta_{rel})]^{1/2}/c \quad (\text{Equation 3.10})$$

The intrinsic viscosity of a polymer is related its molecular weight by the Mark-Houwink equation (Mark, 1938; Houwink, 1941)

$$[\eta] = KM^a$$

(Equation 3.11)

where  $K$  and  $a$  are constants whose values depend upon the 'shape' of the polymer, the solvent used and the temperature of measurement. Once  $K$  and  $a$  for a specific polymer/solvent system have been obtained by calibration against a primary technique such as light scattering, intrinsic viscosity measurements then provide a simple method for subsequent determinations of molecular weight.

### 3.3. CHIROPTICAL TECHNIQUES

The rheological techniques discussed in the previous section of this chapter emphasized their role in describing a biopolymer system in terms of its 'macroscopic' properties. However, rheology gives little information on the 'microscopic' properties of biopolymers, such as molecular structure and conformation. In order to obtain such information, the closely related chiroptical techniques of optical rotatory dispersion (ORD) and circular dichroism (CD) have been frequently utilised due to their critical sensitivity to the three-dimensional geometry of most biological materials. Both chiroptical techniques provide essentially the same information and examine the differences in the interaction of left and right



handed circularly polarized light with the electrons of dissymmetric molecules (Velluz *et al.*, 1965; Djerassi, 1960). Optical rotation arises from the differences in their rates of propagation through the sample, whereas circular dichroism measures the differential absorption. CD thus has the additional requirement of a chromophore that absorbs light in an accessible spectral region.

Experimentally, optical rotation studies measure the angle through which the sample rotates incident plane-polarised light. In plane-polarised light the electric vector is restricted to one plane and its magnitude varies sinusoidally with time. As is shown in Fig. 3.5, plane polarised light is equivalent to the sum of right and left circularly polarised components of equal amplitude ( $E_r$  and  $E_l$ ). When this light passes through an optically active sample, the rate of propagation of the circular components is altered relative to one another and hence the plane of polarisation of the light emerging from the sample is displaced through an angle ( $\alpha$ ) from that entering the sample (Fig. 3.6), i.e. optical rotation has occurred.

Similarly, a chromophore in a dissymmetric molecule absorbs left and right circularly polarised light of the correct wavelength to different extents; the resultant vector is then no longer truly plane polarised but describes an ellipse (see Fig. 3.7). The

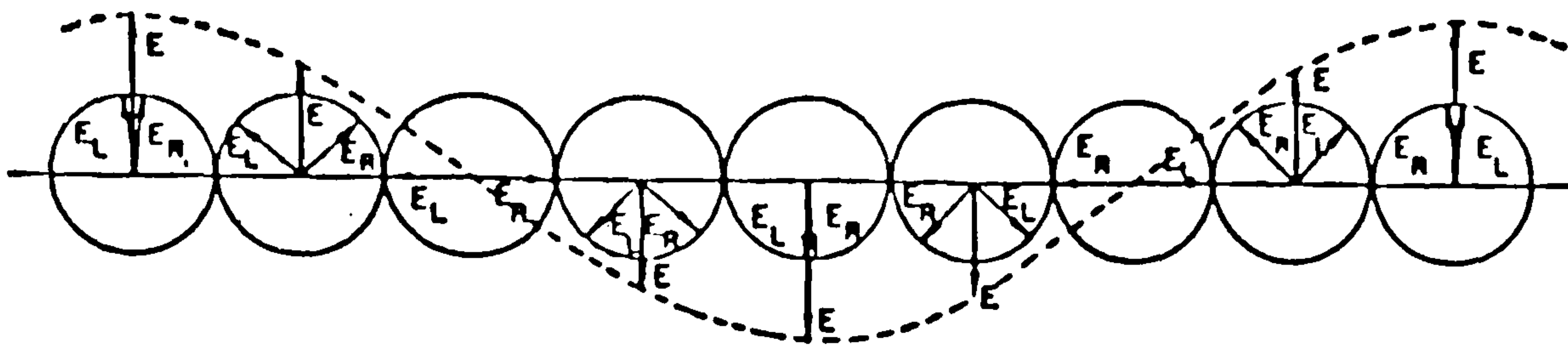


Figure 3.5. Left and right circularly polarized components of plane-polarized light.

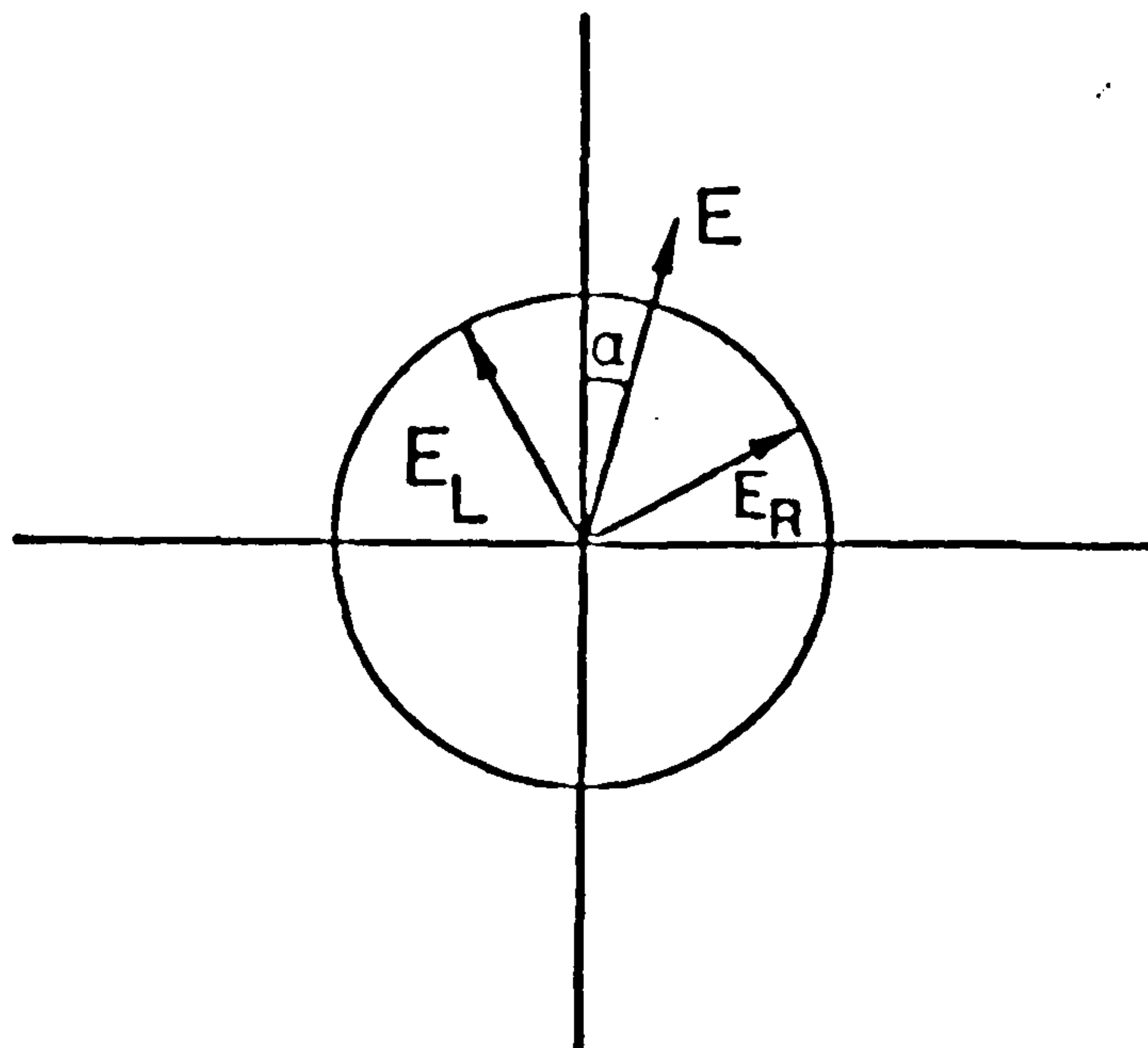


Figure 3.6. Rotation of plane-polarized light resulting from the decrease in propagation rate of one of the circularly polarised components relative to the other when the light passes through an optically active sample.

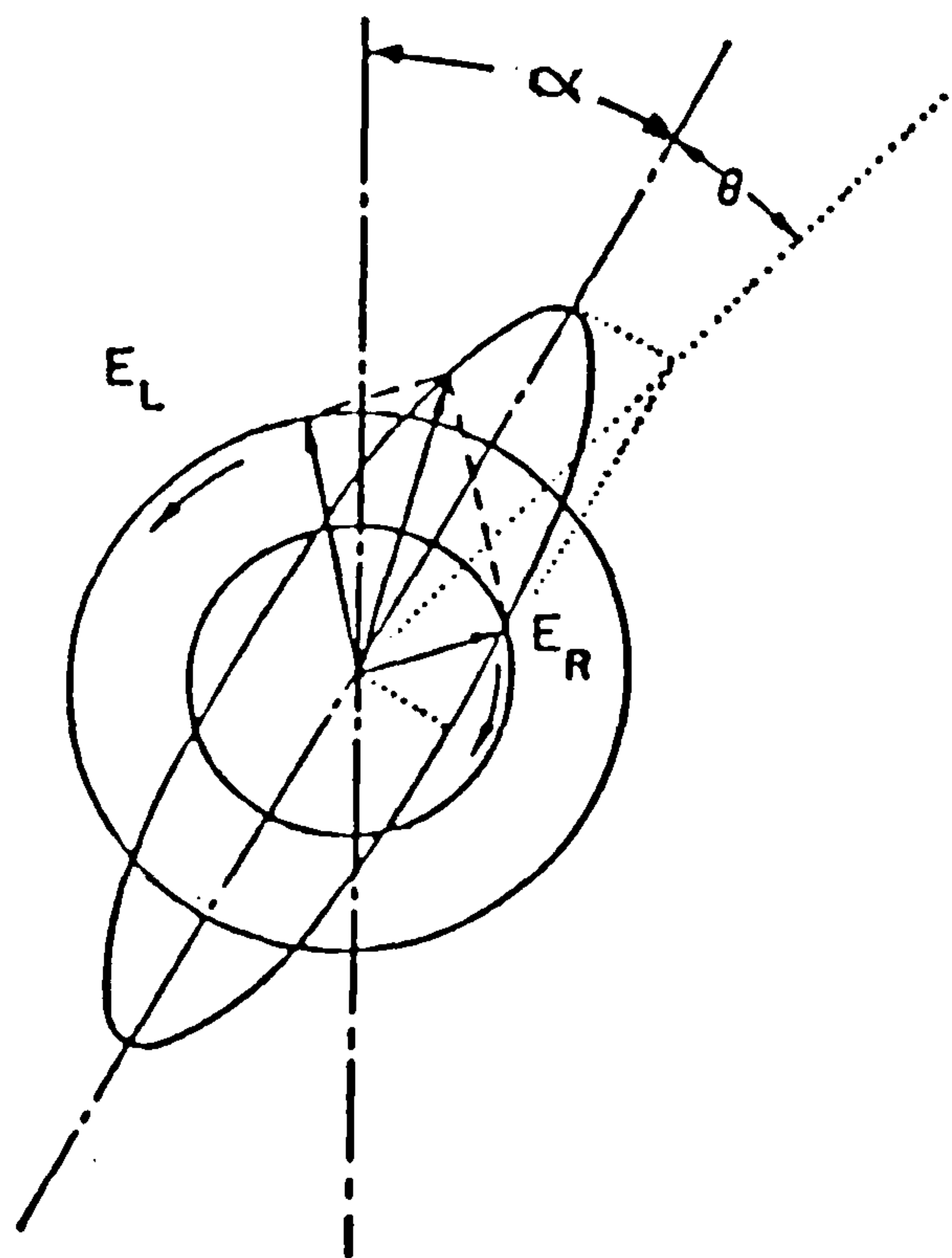


Figure 3.7. Effect of an optically active absorbing sample on plane-polarized light. The effect is not only to rotate the plane of polarization through the angle,  $\alpha$ , but also to cause the light to propagate elliptically.  $\theta$  is the ellipticity.

circular dichroism of a sample is the ratio of the difference in absorption to total absorption.

Alternatively, the angle  $\theta$  known as the ellipticity and defined by the relationship  $\tan \theta = \text{minor axis}/\text{major axis}$  is often quoted.

To remove the effects of concentration and pathlength results can be expressed in molar quantities, i.e. molar **ellipticity**,  $[\theta]$  and molar rotation,  $[\alpha]$

$$[\theta] = \theta M / lc \text{ deg cm}^2 \text{ dmol}^{-1} \quad (\text{Equation 3.12})$$

$$[\alpha] = \alpha M / lc \text{ deg cm}^2 \text{ dmol}^{-1} \quad (\text{Equation 3.13})$$

where  $M$  is the molecular weight of the sample (or mean residue weight for polymers),  $l$  is the pathlength in mm,  $c$  is the concentration in  $\text{g ml}^{-1}$ , and  $\theta$  and  $\alpha$  are the observed **ellipticity** and optical rotation in **degrees**, respectively. An alternative convention still widely used to characterise biopolymer optical rotation, is that of specific rotation,  $[\alpha]$ , defined as

$$[\alpha] = 100\alpha / lc \text{ mdeg. g}^{-1} \text{ dm}^2 \quad (\text{Equation 3.14})$$

where concentration is in  $\text{g}/100 \text{ ml}$  and pathlength is in  $\text{dm}$ . The two conventions are related by:

$$[\alpha] = [\theta] M / 100 \quad (\text{Equation 3.15})$$



The actual magnitude of optical rotation varies with the wavelength of light used (this variation is known as optical rotatory dispersion). The fundamental band forms for the two chiroptical techniques (ORD and CD) are illustrated in Fig. 3.8 for a single optically active transition. If the three parameters, position,  $\lambda_0$ , maximum intensity,  $[\theta]_0$ , and width,  $w$  of the CD band are known, then, using the Gaussian and Kronig-Kramers transform equations (Equations 3.16 and 3.17 respectively; Djerassi, 1960), it is possible to calculate the contribution of that transition to the ORD and CD spectra at any wavelength ( $\lambda$ ).

$$[\theta]_{\lambda} = [\theta]_0 e^{-(\lambda - \lambda_0)^2 / w^2} \quad (\text{Equation 3.16})$$

$$[\phi]_{\lambda} = \frac{2[\theta]_0}{\sqrt{\pi}} \left( e^{-(\lambda - \lambda_0)^2 / w^2} \int_0^{(\lambda - \lambda_0)/w} e^{x^2} dx - \frac{w}{2(\lambda + \lambda_0)} \right) \quad (\text{Equation 3.17})$$

The fundamental band form for ORD (Fig. 3.8) is complex, with two equal and opposite lobes on either side of the band centre (where CD attains its maximum values but optical rotation passes through zero). The sign of the higher-wavelength lobe (and therefore of its contribution to optical rotation at long wavelengths) is the same as that of the corresponding CD band. The ORD spectrum over the wavelength range of an electronic transition, with its characteristic minimum

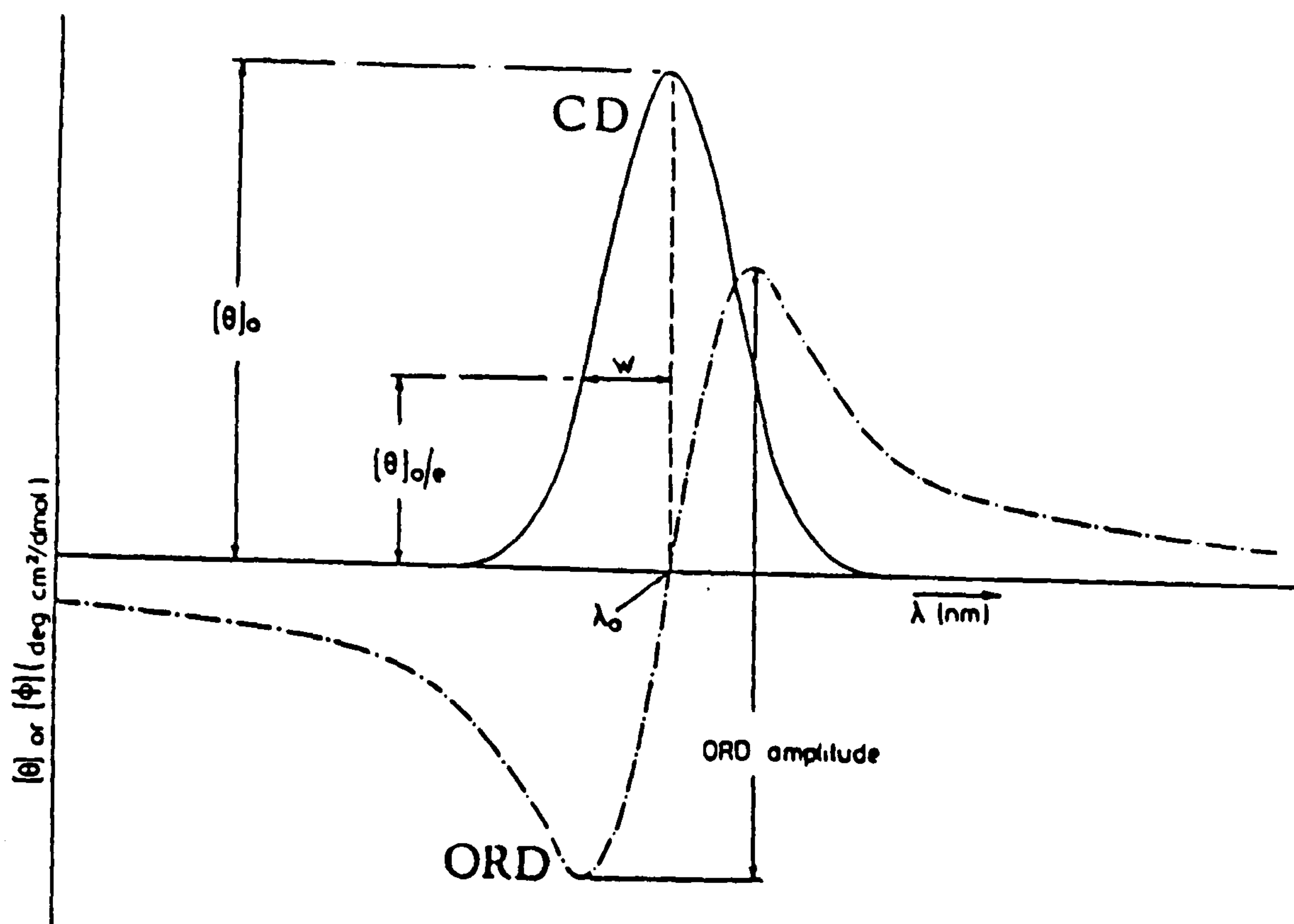


Figure 3.8. Fundamental spectral band form of CD and ORD for a single optically active transition. Both CD molar ellipticity  $[\theta]$  and ORD molar rotation  $[\phi]$  at any wavelength can be determined completely (Equations 3.16 and 3.17, respectively) by the three parameters: position  $\lambda_0$ , intensity  $[\theta]_0$  and width  $w$  (= half-width at  $1/e$  of maximum intensity).

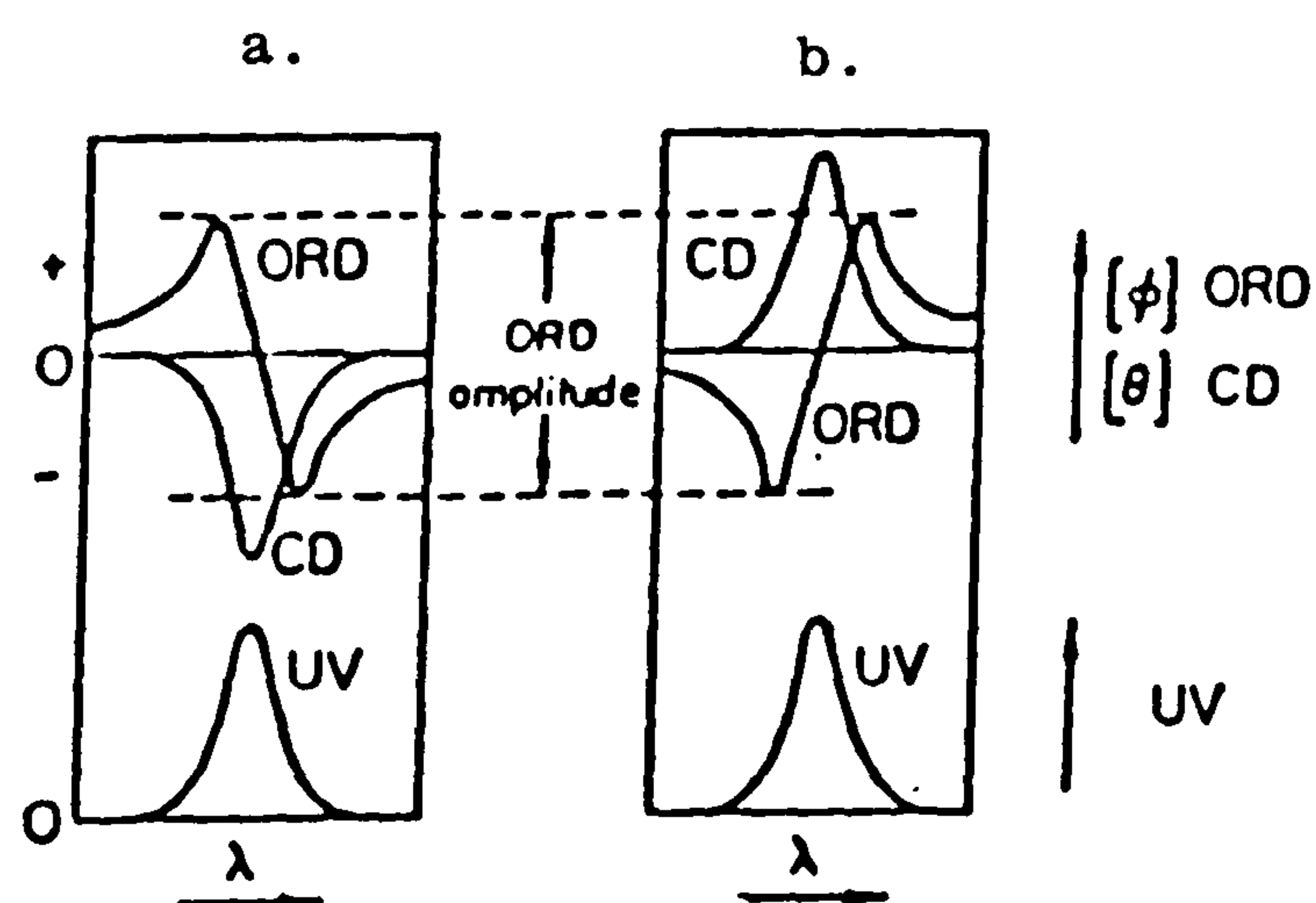


Figure 3.9. Idealized U.V. CD and ORD spectra for an isolated chromophore: a) - laevorotatory molecule (negative Cotton effect); b) dextrorotatory molecule (positive Cotton effect).

and maximum values, was known historically as a 'Cotton curve' (either positive or negative depending on the sign (see Fig. 3.9)) and the higher-wavelength portion, where the absolute value of optical rotation decreases monotonically, was described as a 'plain curve'. In contrast to CD, which drops off rapidly to zero on either side of the band centre, optical rotation values remain within an order of magnitude of the maximum ORD to long wavelengths, and can therefore be used to monitor changes in the optical activity of transitions inaccessible by CD. The wavelength dependence of optical rotation in the 'plain curve' region can be described, with reasonable precision, by the Drude equation (Equation 3.18), a limiting form of the Kronig-Kramers relationship (Equation 3.17) for  $\lambda - \lambda_0 \gg w$ .

$$c/[a] = \lambda^2 - \lambda_0^2 \quad (\text{Equation 3.18})$$

where  $c$  is a constant.

Plots of  $1/[a]$  against  $\lambda^2$  (Drude plots) are therefore approximately linear, and can be used for calculation of optical rotation at wavelengths other than those accessed experimentally (See Chapter 6).

For polysaccharides, the conformation-sensitive backbone transitions are centred in the vacuum ultra-violet, below the lower wavelength limit of current commercial CD equipment ( $\sim 185$  nm), and are therefore usually monitored indirectly by optical



rotation measurements at higher wavelengths. Only when polysaccharides have accessible u.v. chromophores such as carboxyl groups can the CD behaviour be observed directly.

For proteins, however, the transitions of the polymer backbone are in a readily-accessible spectral region (250 nm and below) and CD studies are now widely acknowledged as the most informative spectroscopic probe of protein secondary structure in solution. The CD spectra obtained arise from the  $n-\pi^*$  and  $\pi-\pi^*$  electronic transitions of the peptide group, with their precise shape and position depending on the molecular environment of the chromophores (i.e. conformation of the protein backbone). The well-known conformation states of proteins, that is,  $\alpha$  helix,  $\beta$  sheet and random coil give rise to characteristic band shapes (Fig. 3.10) and it is possible, by linear combination of reference spectra, using an iterative computer method, to match observed protein spectra, thus giving a reasonable estimate of the fractions of  $\alpha$  helix,  $\beta$  sheet and disordered sequences present.

In the case of gelatin, the CD spectra above the melting temperature is typically random coil having a large negative peak below 200 nm. The triple helical ordered state of gelatin, however, shows its own characteristic CD spectrum, and gel formation is accompanied by a large increase in the magnitude of the

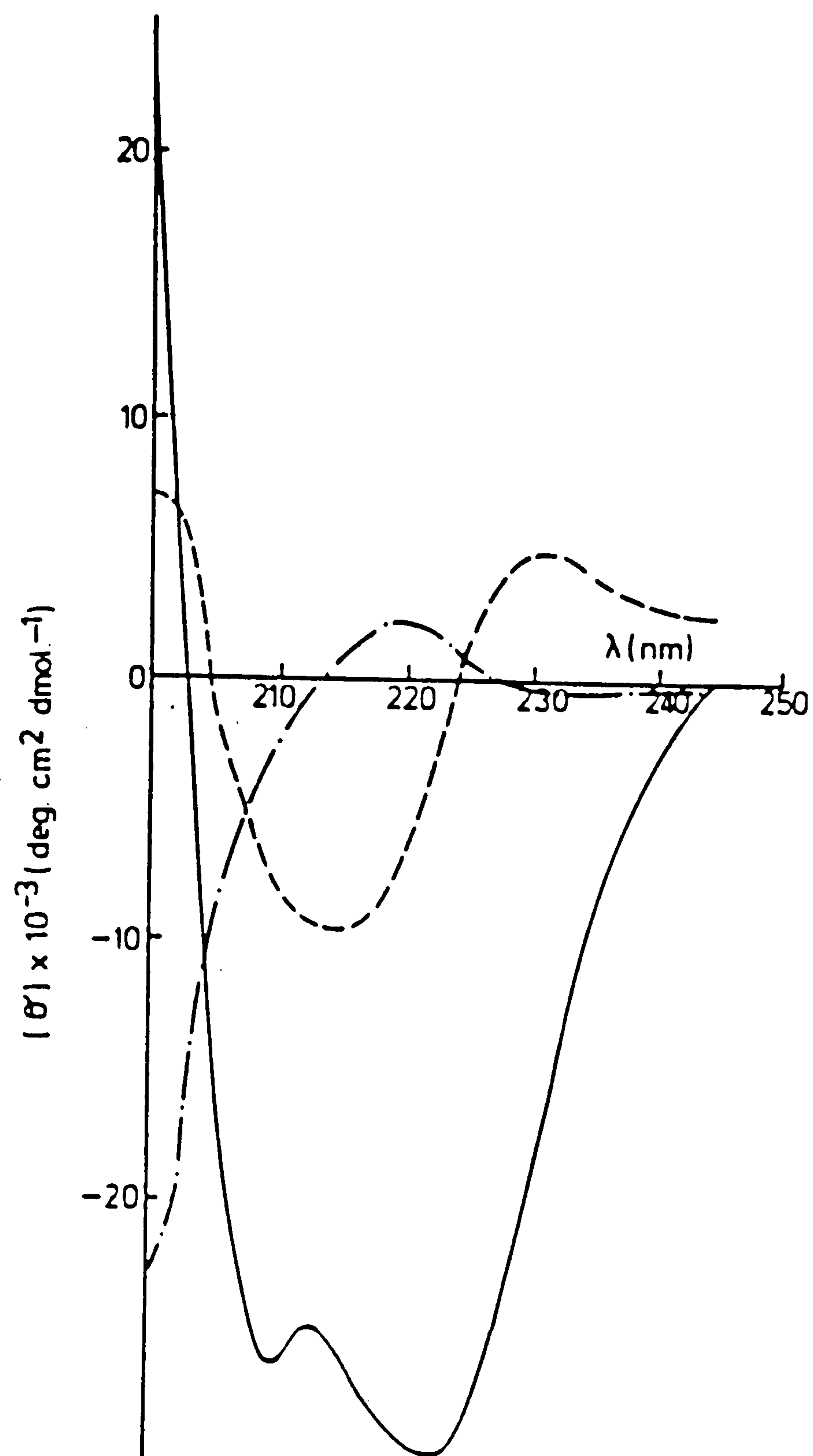


Figure 3.10. CD behaviour of peptide residues in different conformational sequences within globular proteins of known secondary structure,  $\alpha$ -helix (—),  $\beta$ -sheet (----) and disordered (-·-·-) sequences (Chen *et al.*, 1972).

optical rotation signal at higher wavelengths. This increase in optical rotation has often been used in calculating the helix-fractions existing in gelatin gels (See Chapter 6.3).

#### 3.4. DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) has frequently been used as a method for obtaining thermodynamic information on biopolymer conformation changes. For polysaccharides, it has been one of the major physical techniques utilised in studies on the gelatinization of starch, and in protein denaturation studies many of the conformational changes have been characterised in terms of their melting profiles.

Essentially, the technique of DSC measures the absorption or release of heat as the sample under test is either heated or cooled at a constant rate. In the absence of any phase transition a monotonic line is obtained, the slope of which is a measure of the heat capacity of the sample. In most DSC apparatus, however, it is not the actual amount of heat absorbed that is measured, but the excess power input (i.e. the amount of excess heat per second) required to heat or cool the sample relative to a reference of approximately the same heat capacity. Under these circumstances, a flat



baseline should be obtained, the value of the ordinate giving a measure of the heat capacity of the sample relative to that of the reference.

If, however, the sample undergoes a phase transition, additional heat absorption or release occurs due to the latent heat of the transition, which shows up as a peak in the DSC profile; the position of the peak gives the temperature of the transition directly. Fig. 3.11 illustrates a typical DSC profile for a substance which takes in energy on changing phase. Quantitatively, it is not only possible to obtain an accurate measure of the transition temperature, but also to determine with high precision, the enthalpy of the transition,  $\Delta H$ .

Consider the transition occurring in Fig. 3.11, in which a substance changes from some low temperature state L to some higher temperature state H. The heat capacity ( $C_p^L$ ) of the lower temperature state can be obtained by extrapolating the line AB back to the heat flow axis, and similarly the heat capacity ( $C_p^H$ ) of the higher temperature state can be obtained by extrapolating the line CD. The enthalpy ( $\Delta H$ ) of the transition on changing from L to H is equivalent to the area of the peak, BMCRS, multiplied by the time difference between points B and C (obtained from the scanning rate). The differences in heat capacities between the two states ( $\Delta C_p$ ) can be obtained from the magnitude of RS.

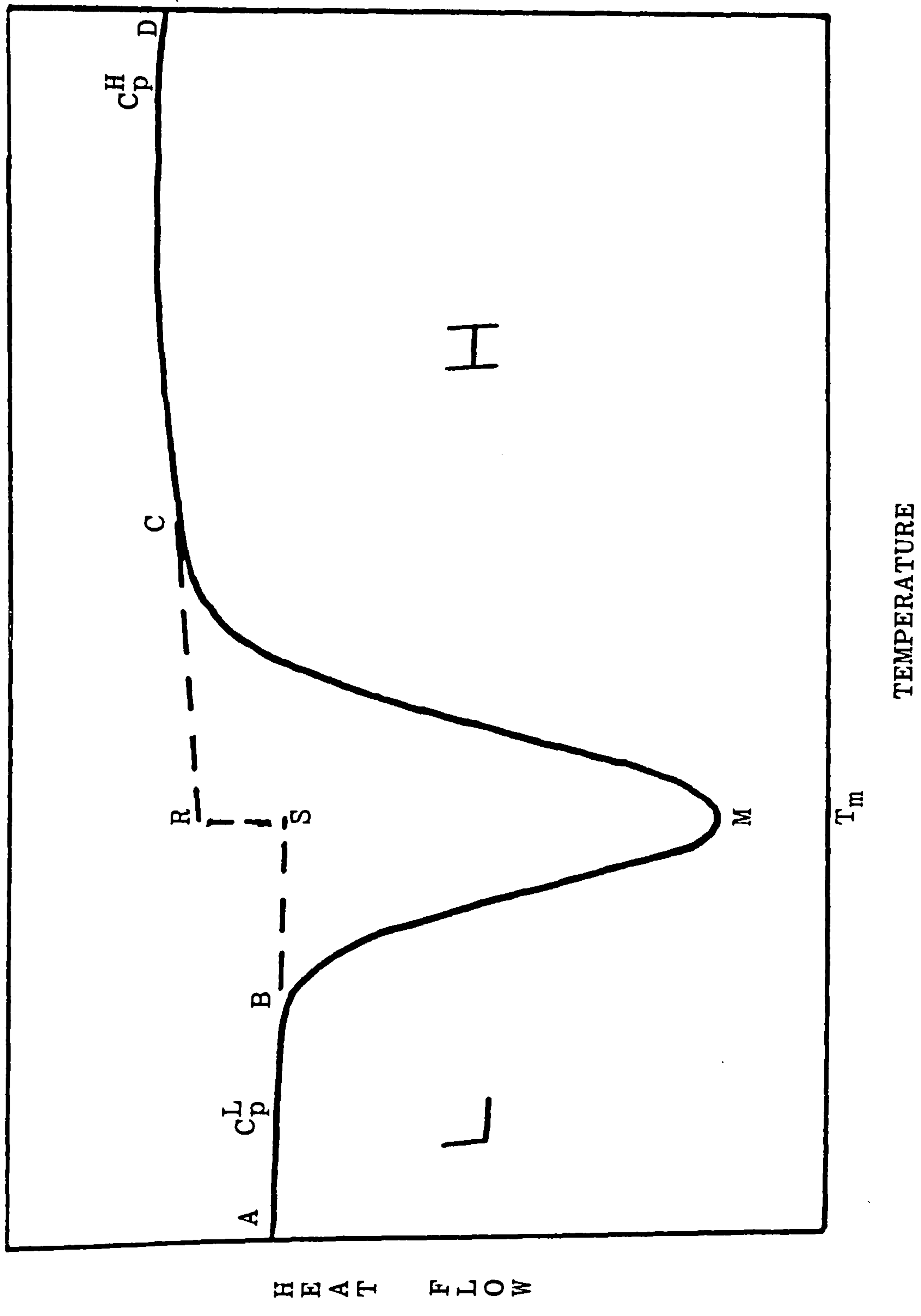


Figure 3.11. Illustration of a DSC profile for a substance which takes in energy on changing phase. The enthalpy of the transition can be obtained from the magnitude of the area BMCRS.

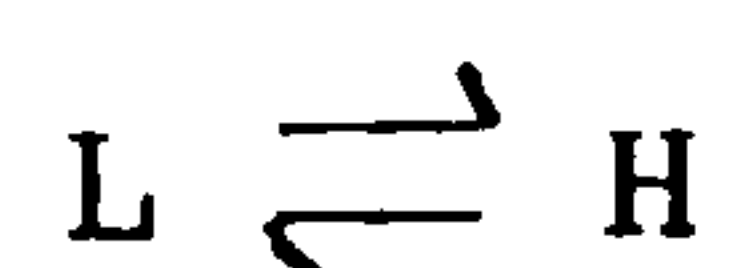
Obviously, the extrapolations of the baseline, BS and CR, are necessary to obtain an estimate of both  $\Delta H$  and  $\Delta C_p$ . When the transition is sharp or the heat capacities of the higher and lower temperature states are much the same, there is little problem in extrapolating the baseline, provided calorimetric data above and below the transition region has been obtained. If, however, the transition is broad, with a large difference in heat capacity between the two states, then extrapolation becomes increasingly difficult, and can be the major source of error in the results obtained from DSC.

The earlier generations of differential scanning calorimeters were designed primarily for studying phase changes with large associated changes in enthalpy (e.g. melting of solids) and as such were not required to have high sensitivity; this type of calorimeter utilised small sample volumes and fast scanning rates. The application of differential scanning calorimetry to measurement of thermal properties of biopolymers, where the enthalpies associated with conformational changes are much smaller than those involved in phase transitions, required the development of much more sensitive calorimeters. The latest calorimeters achieve their increased sensitivity through the use of larger sample volumes and require slower heating rates to allow thermal equilibration of the sample throughout



the temperature scan. Using these high precision calorimeters, the thermal denaturation profiles of many proteins have been determined, with the shapes of the profiles indicating the nature of the multistage denaturation processes which proteins may undergo, i.e. different DSC peaks corresponding to specific conformation changes within the same protein molecule (Fig. 3.12).

The actual shape of a DSC peak can also provide information about the transition being monitored. If the transition is a two state equilibrium process with only insignificant concentrations of intermediate states, e.g.



then the equilibrium constant for the process may be written as:

$$K = [H]/[L] \quad \text{(Equation 3.19)}$$

The temperature-dependence of the equilibrium constant for a chemical reaction is related to the associated enthalpy change by the van't Hoff equation (Equation 3.20). Thus the apparent van't Hoff enthalpy change,  $\Delta H_{vH}$ , can be determined by plotting the logarithm of  $K$  against the reciprocal of temperature.

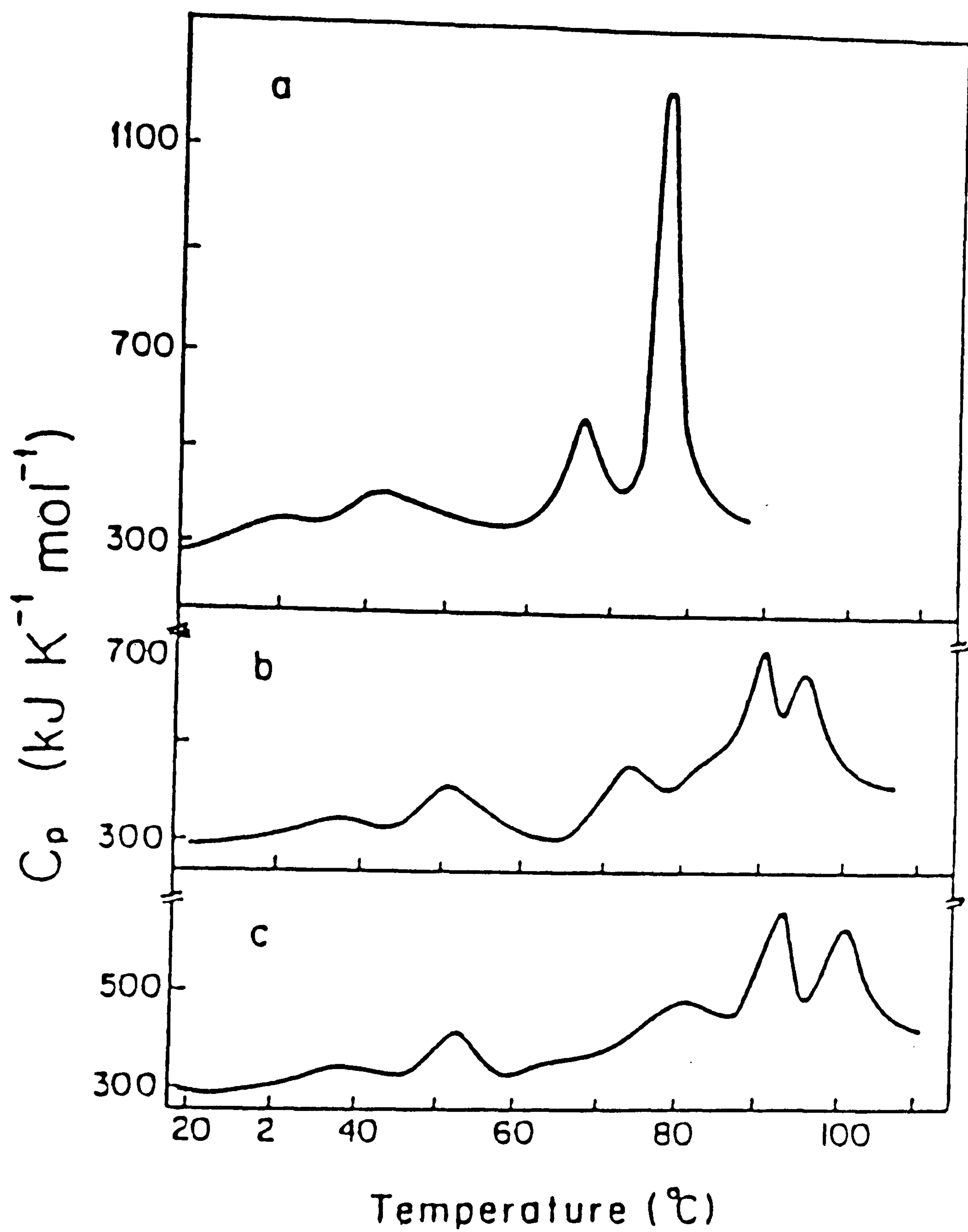


Figure 3.12. The heat capacity function of the protein paramyosin in 10 mM glycine buffer at pH 2 (a), pH 3 (b) and pH 3.5 (c) showing a multi-stage denaturation process. (From Privalov, 1982).

$$\ln K = \Delta H_{vh}/RT + \text{constant} \quad (\text{Equation 3.20})$$

The value of  $K$  at temperatures throughout the transition can be obtained from an integrated DSC peak.

For reactions of small molecules,  $\Delta H_{vh}$  is the same as the calorimetric enthalpy change,  $\Delta H_{cal}$ . For co-operative transitions of biopolymers, however,  $\Delta H_{vh}$  is usually much greater than  $\Delta H_{cal}$ , the ratio of one to the other giving a measure of the apparent number of residues undergoing conformational change as a concerted process. Accordingly, the co-operativity of the process can be estimated from the width of the DSC peak, with narrow peaks indicating a large degree of co-operativity and broad peaks reflecting less co-operative transitions.

Calorimetry is therefore a very valuable tool for determining the thermodynamic parameters of biopolymer conformation change. Although by itself it is mechanistically uninformative, in conjunction with other physical techniques it is capable, in many cases, of providing further evidence either in support of or against a postulated conformational mechanism.



## CHAPTER 4: METHODS AND MATERIALS

### 4.1. METHODS

#### 4.1.1. Preparation of samples

Preparation of all the gelatin solutions was achieved using the following procedure. The raw gelatin was allowed to stand in the solvent (usually 0.1M sodium chloride) for three hours at room temperature allowing the gelatin to swell. The swollen gelatin was then heated in a water bath at 50°C, with occasional stirring, for a further thirty minutes to dissolve the gelatin and ensure the molecules were in a random conformational state. Following this, the pH of the solution was adjusted to pH7 using concentrated sodium hydroxide solution or concentrated hydrochloric acid. All samples had sodium azide (0.02% w/w) present to prevent microbial growth. Any dilutions of the solutions to lower concentrations were made using a stock solution containing sodium chloride (0.1M) and sodium azide (0.02% w/w) at pH7.

#### 4.1.2. Physical Methods

##### (1) Rheological and Hydrodynamic Investigations

Mechanical spectra and rigidity measurements were performed on a Sangamo Viscoelastic Analyser using a cone and plate geometry of cone angle 2 deg and diameter 5 cm. The sample, at 45°C, was placed on the plate of the instrument, the temperature of which was

controlled by a Haake F4-C circulating water bath and monitored by an attached thermocouple. When melting gelatin gels, at least twenty minutes was allowed for thermal equilibration of the sample after any temperature change, before readings were taken.

Intrinsic viscosity measurements on dilute solutions were carried out using the Ubbelohde type of capillary viscometer submerged in a water bath, the temperature being accurately held at  $45 \pm 0.2$  C. Initially, a low-shear concentric cylinder viscometer was used, but gave values the same as those from capillary viscometry, indicating that, at the gelatin concentrations used, shear-thinning effects were negligible, even at the high shear rates generated in the capillary. Since the capillary method is faster and more precise, it was therefore adopted for all subsequent measurements. All dilutions to lower concentrations were carried out inside the viscometer.

## (2) Differential Scanning Calorimetry

A Setaram micro DSC "batch and flow" calorimeter was used to obtain the melting profiles of the gelatin gels. The sample pan was prepared by accurately weighing the pan and lid, filling the pan with the gelatin solution at 45 C, sealing the pan with a small spanner and then weighing (to within 0.1 mg) the sealed pan. Being a 'thermal balance' calorimeter, it was necessary to prepare a reference pan containing exactly the same



amount of solvent as was present in the sample pan. This was to ensure minimisation of the differences in heat capacity between the sample and reference pans thus producing the flattest possible baselines. Plastic gloves were worn at all times when handling the pans to prevent contamination with grease deposits, which could, if present, invalidate the results. The pans were placed in the machine at  $45^{\circ}\text{C}$  and held at this temperature for 30 minutes before being quenched to the experimental temperature at the maximum possible cooling rate. After gelling, the sample was melted and the melting profile obtained. The enthalpy of melting was determined either by measuring the area of the peak with a planimeter or by weighing the peak and comparing the value with that of a corresponding piece of chart paper whose 'enthalpy value' was known from the heat flow and temperature axes.

### (3) Optical Rotation Measurements

Measurement of optical rotation was carried out on a Perkin-Elmer 241 Polarimeter, using jacketed cells of pathlengths 1 and 10 cm. As in the other physical measurements, the gelatin solutions were injected into the cells from a stock solution held at  $45^{\circ}\text{C}$ . Temperature control of the sample was achieved using a circulating water bath controlled to within  $0.5^{\circ}\text{C}$  over the temperature range studied, the actual sample temperature being monitored by a thermocouple. In melting experiments a 20 minute equilibration period was



allowed before readings were taken, and in temperature quench experiments the temperature was quickly altered by switching to a second water bath at the desired quench temperature, prior to observing the change in optical rotation with time. Optical rotation readings were taken at various wavelengths (255, 365, 436, 546 and 578 nm), the actual wavelength used being decided by the concentration of the gelatin solution and the length of polarimeter cell involved.

#### (4) Circular Dichroism Studies

CD spectra were recorded on a Jobin Yvon Auto Dichrograph Mark V Spectropolarimeter using a 1 s integration period. Gelatin solutions were injected into the CD cells, which were maintained at the required temperature by means of a circulating water bath, circulating water through the cell compartment. Cells of pathlengths in the range 0.05 mm to 0.2 mm were used, the choice of cell being determined primarily by the wavelength range under study. At higher wavelengths (260-210 nm), higher gelatin concentrations had to be used than at lower wavelengths (230 - 180 nm), where the magnitude of the CD necessitated the use of very low gelatin concentrations. Spectra were recorded at 1 nm intervals, with accumulation of at least 15 scans to improve the signal-to-noise ratio. Cell blanks recorded in the same way were then subtracted. Once again, 20 minutes was allowed for thermal equilibration in gel-melting experiments.

## 4.2. MATERIALS

### 4.2.1. General Materials

General laboratory reagents such as sodium azide, sodium chloride etc., used throughout this work, were all of analar grade and obtained from BDH Chemicals Ltd., Poole, Dorset, UK.

### 4.2.2. Gelatins and their Characterisation

The gelatin samples used were kindly supplied by Sanofi Bio-industries Ltd., and consisted of eight samples, four of which were extracted from ossein pretreated with lime, the other four being obtained from the acid pretreatment process on pigskin. The four samples of each pretreatment process represented the 1st, 2nd, 3rd and 4th cuts of the extraction stages and were therefore coded according to their source and pretreatment (acid pigskin - AP; limed ossein - LO) and their cut number (1,2,3,4). For example, AP-3 would be the code for the third cut of the gelatin produced from acid pretreatment of pigskin. The gelatin samples were not further purified or fractionated in any way but used directly as supplied. The remainder of this chapter discusses the characterisation of the eight samples and presents the results of various tests/characterisation procedures to which they were subjected.



#### 4.2.2.1 Rheological Properties of the Gelatin Samples

The intrinsic viscosities of the samples were measured using capillary viscometry as described in section 4.1.2. Figs. 4.1a and 4.1b show the extrapolations used to determine the intrinsic viscosities listed in Table 4.1. For the limed ossein samples LO-1, LO-2 and LO-3, the intrinsic viscosity values are relatively large, the value increasing slightly with increasing extract number. LO-4, however, the final extract sample, has a vastly reduced value of intrinsic viscosity, indicating a molecular weight considerably lower than those of the first three extracts. For the acid pigskin gelatins, AP-1 has the highest intrinsic viscosity with AP-2 having a slightly lower value. The third and fourth cuts of acid pigskin, AP-3 and AP-4, have much smaller intrinsic viscosity values than the first two extracts. Viscosity average molecular weights (Table 4.1) were estimated from the intrinsic viscosity values using Mark-Houwink parameters of  $K = 1.15 \times 10^{-3} \text{ dl g}^{-1}$  and  $a = 0.52$  (derived as an overall best fit to the various sets of experimental data collated by Veis, 1964).

A mechanical spectrum of each of the eight samples was recorded on a 5% w/w gel formed by curing at 5°C for 16 hours. The results of this test are presented in Fig. 4.2a and Fig. 4.2b which show the same general shape for all samples (i.e. typical gel-like behaviour with  $G'$  being constant and higher than  $G''$  at all accessible frequencies, and  $\eta^*$  decreasing with a gradient of



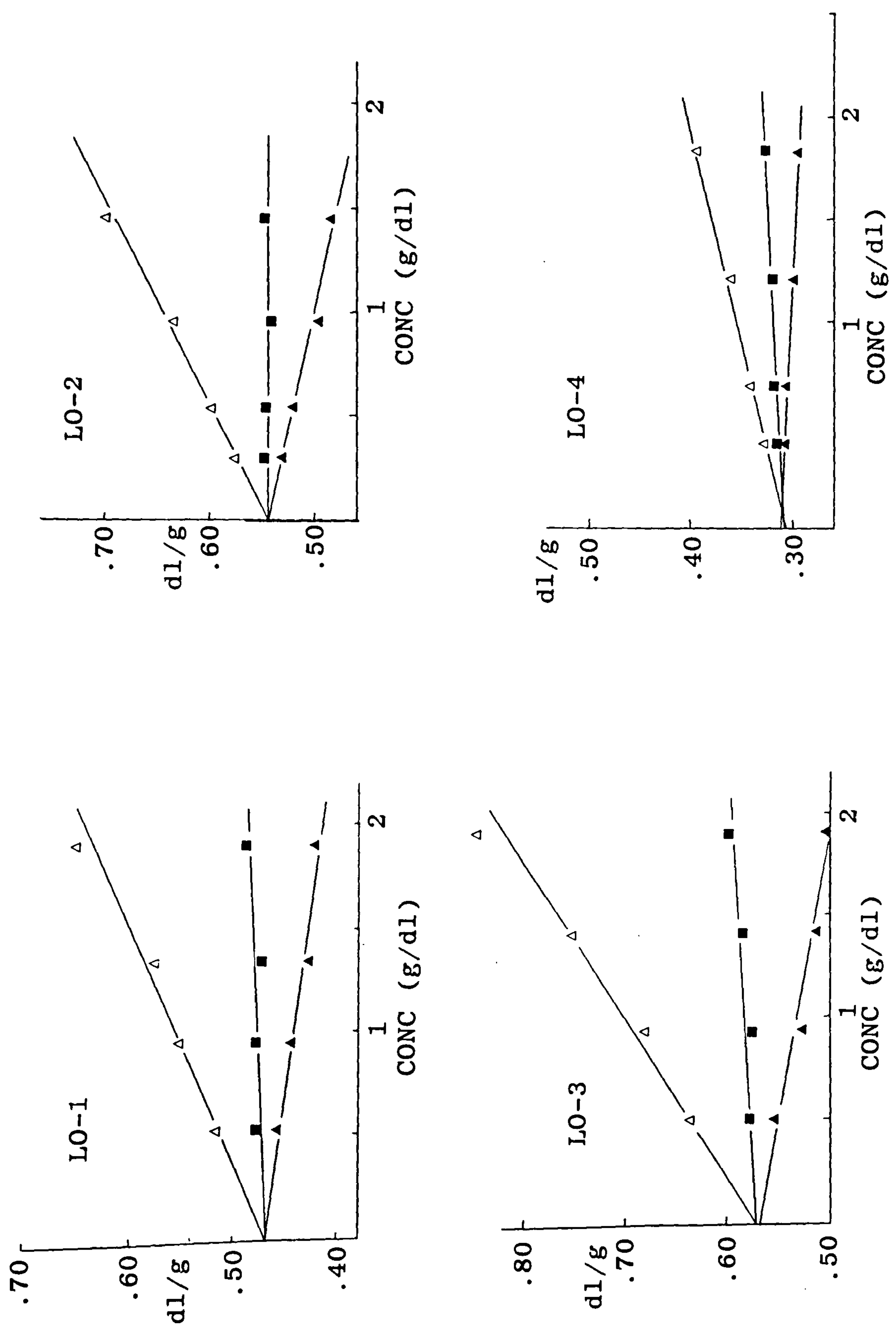


Figure 4.1a The extrapolations used in the determination of the intrinsic viscosity values listed in Table 4.1. for the limed ossein samples. (Huggins plots ( $\Delta$ ), Kraemer plots ( $\blacksquare$ ), single point plots ( $\blacktriangle$ )).

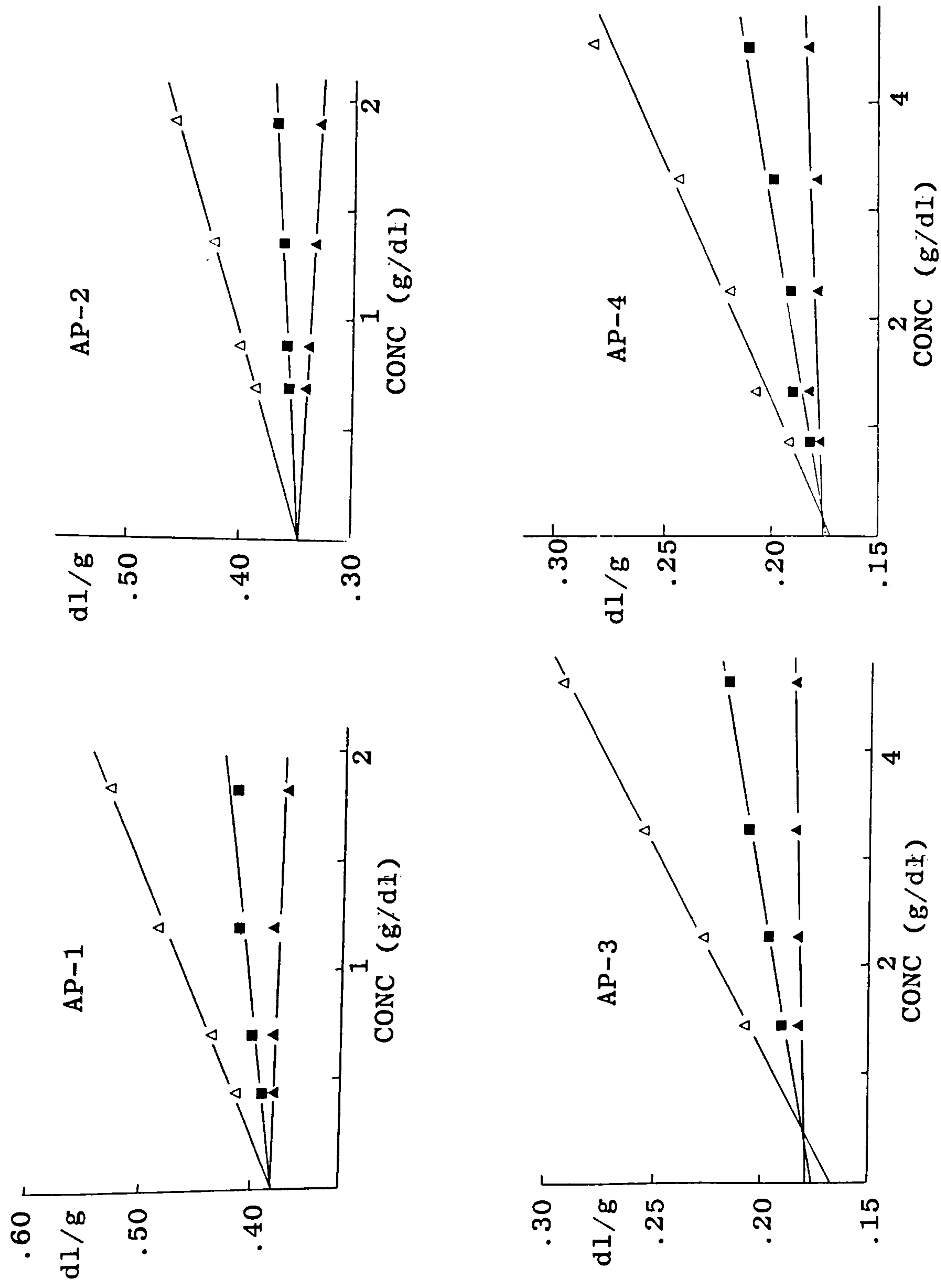


Figure 4.1b The extrapolations used in the determination of the intrinsic viscosity values listed in Table 4.1. for the acid pigskin samples (Huggins plots ( $\Delta$ ), Kraemer plots ( $\blacktriangle$ ), single point plots ( $\blacksquare$ )).

TABLE 4.1.

EXPERIMENTAL VALUES OF VARIOUS PARAMETERS OBTAINED  
FOR THE EIGHT GELATIN SAMPLES

SAMPLE	$[\eta]$ (dl/g)	$M_v^a$ (kD)	$G'^b$ (dyn/cm <sup>2</sup> )
LO-1	0.470	105	45500
LO-2	0.545	140	44000
LO-3	0.570	152	36000
LO-4	0.305	46	18500
AP-1	0.380	70	51000
AP-2	0.345	58	46000
AP-3	0.165	14	12000
AP-4	0.170	15	11500

<sup>a</sup> using values of  $K = 1.15 \times 10^{-3} \text{ dl g}^{-1}$  and  $a = 0.52$  obtained as described in the text.

<sup>b</sup>  $G'$  measured on gels (50 mg ml<sup>-1</sup>) after being cured at 5°C for 16 hours.



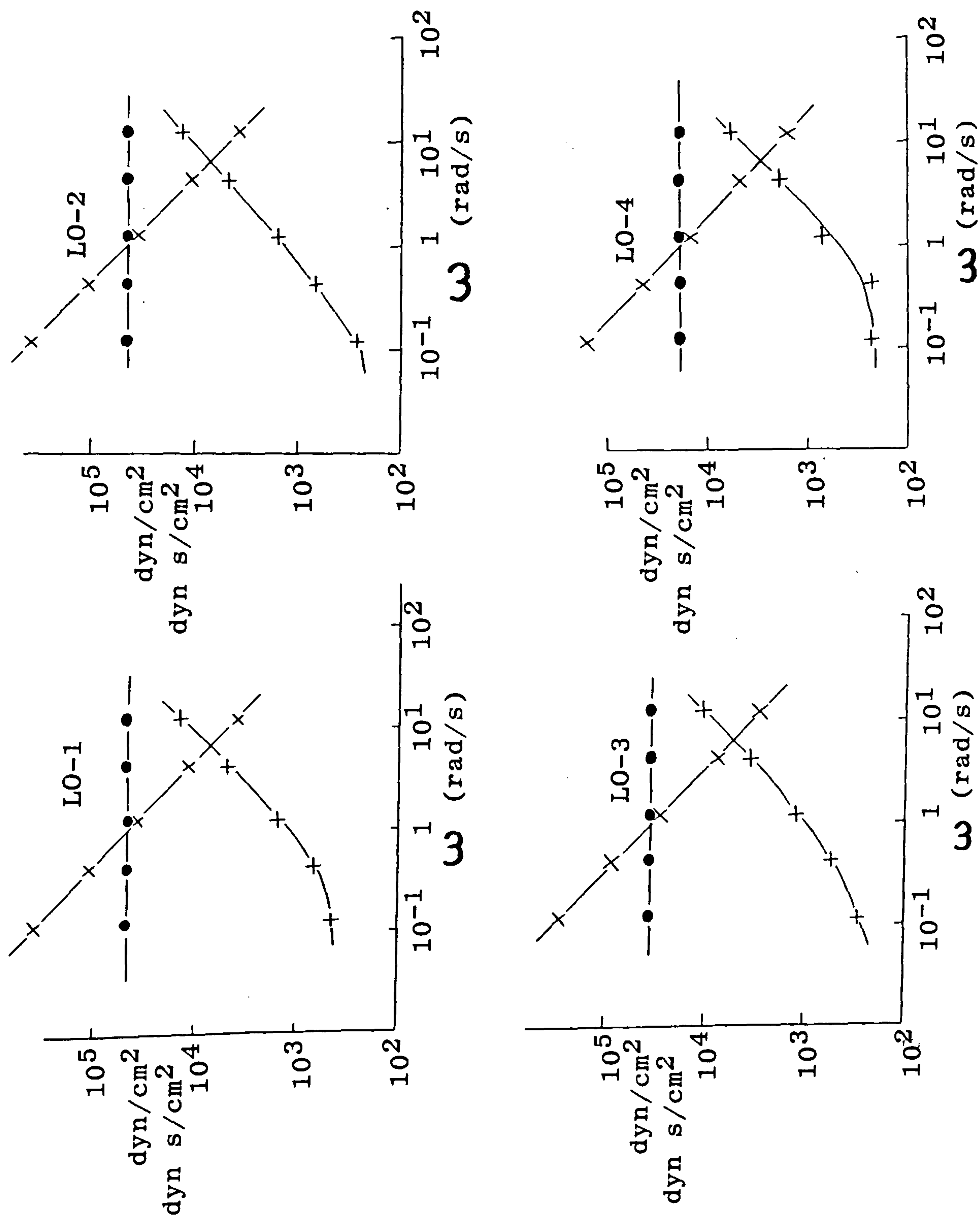


Figure 4.2a Mechanical spectra illustrating the typical gel-like variation in  $G'$  (●),  $G''$  (●) and  $\eta^*$  (X) with frequency of oscillation ( $\omega$ ) for 50 mg  $\text{ml}^{-1}$  limed ossein gelatin gels. Measurements were performed on gels prepared by quenching the warm gelatin solution to  $5^\circ\text{C}$  for 16 hours.

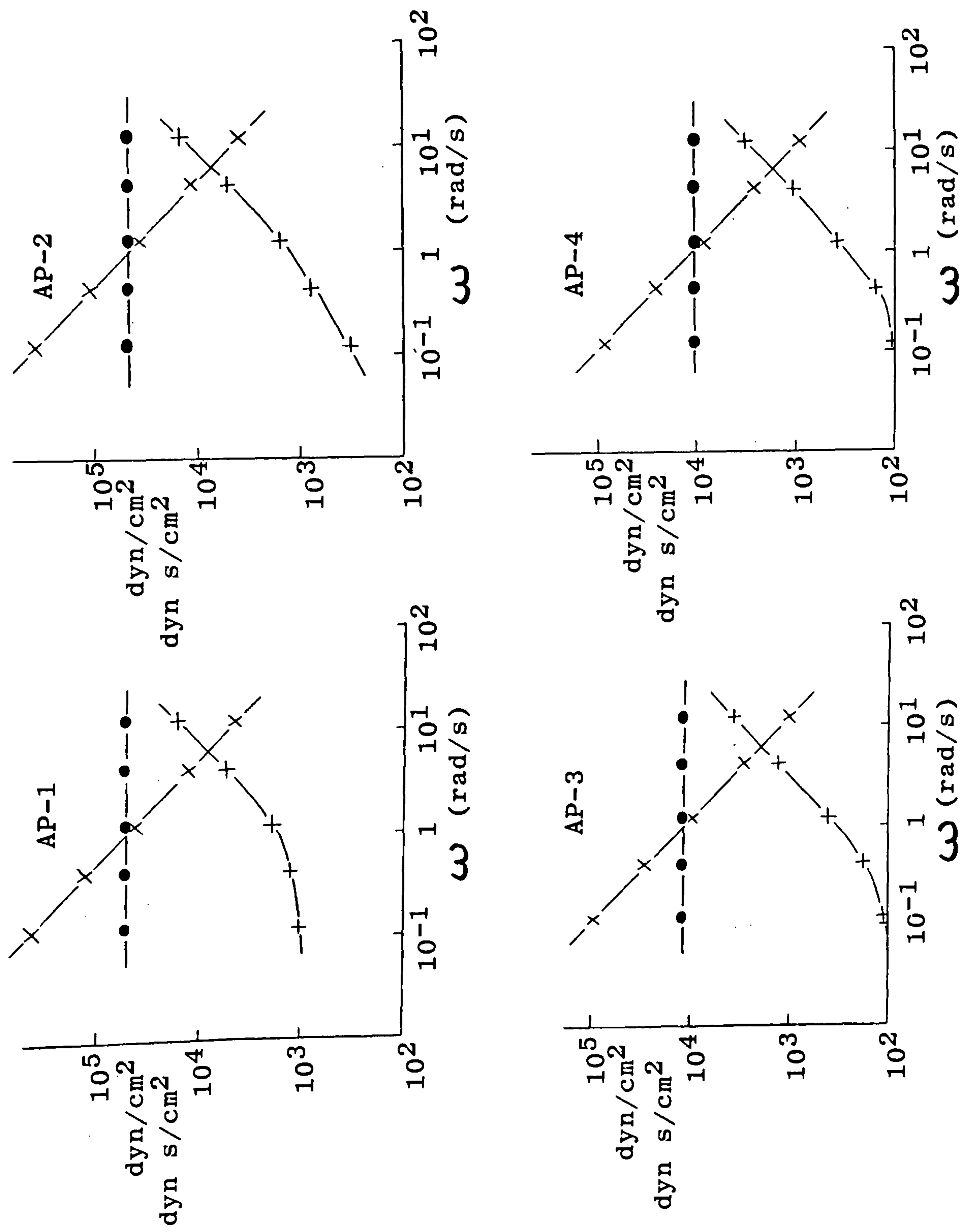


Figure 4.2b Mechanical spectra illustrating the typical gel-like variation in  $G'$  (●),  $G''$  (+) and  $\eta^*$  (X) with frequency of oscillation ( $\omega$ ) for 50 mg ml<sup>-1</sup> acid pigskin gelatin gels. Measurements were performed on gels prepared by quenching the warm gelatin solution to 5°C for 16 hours.

approximately -1). The actual values of  $G'$  (Table 4.1) show no direct relationship with the molecular weights obtained from the intrinsic viscosity data. Samples LO-1 and LO-2, of similar molecular weight, form gels with similar storage moduli but sample LO-3, which has a slightly higher molecular weight, gives a lower value for  $G'$ . Sample LO-4, which has a considerably lower molecular weight, forms gels with much lower storage moduli than any of the higher molecular weight samples. The acid pigskin gelatins show a closer relationship between  $G'$  and molecular weight. The two samples of low molecular weight, AP-3 and AP-4, form gels with much lower storage moduli than AP-1 and AP-2, and also, sample AP-2 which has a slightly lower molecular weight than AP-1, forms gels with a slightly reduced  $G'$ .

#### 4.2.2.2. Molecular Weight Distributions of the Gelatins

The molecular weight distribution within each of the eight samples was investigated using the techniques of gel permeation chromatography (GPC) and polyacrylamide gel electrophoresis (PAGE).

#### Gel Permeation Chromatography

The principle behind the technique of GPC is to fractionate the sample under investigation according to the size of the molecules present. The separating medium is a slurry of solid porous particles packed in a column. It is first equilibrated by pumping through the buffer solution used to dissolve the sample. A small amount of sample is then



introduced into the column and pumped through, along with the buffer. Separation of the different molecular weights present in the sample results from their ability to penetrate, to different extents, into the pores of the stationary phase. Very small molecules can enter all the pores of the column packing material and are eluted last, whereas very large molecules are excluded from the pores and are thus eluted first. Molecules of intermediate size penetrate the stationary phase to different extents, thus being retained on the column for different lengths of time (somewhere between that of the very large molecules and that of the very small molecules).

The GPC apparatus which was used to characterise the molecular weight profiles of the eight gelatin samples consisted of a jacketed column of length 1 m and internal diameter 0.9 cm, packed with a separating medium of Toyopearl HW 55 S. An HPLC system was used to pump the buffer (0.2M disodiumhydrogen orthophosphate in 0.3M NaCl) through the column at a flow rate of 0.5ml/min, the temperature of the column being maintained above 45°C at all times by means of a circulating water bath. 20µl portions of 0.5% gelatin solutions were introduced into the column by means of a 20µl sample loop. Prior to injection the sample was degassed and filtered through a 0.45µm filter. Eluent detection was carried out by monitoring the u.v. absorption of the eluent at 220 nm (the wavelength of maximum absorption of the peptide bond).

The elution profiles of the limed ossein and acid pigskin gelatin samples are presented in Fig. 4.3a and 4.3b respectively. Samples LO-1, LO-2 and LO-3 contain three specific molecular weight fractions, presumably containing the  $\alpha$ ,  $\beta$  and  $\gamma$  components of the gelatin. The amount of high molecular weight material present increases on going from the first to the third extract and LO-1 contains the highest proportion of  $\alpha$  chains. Sample LO-4 on the other hand, has a more symmetrical molecular weight distribution, with no specific molecular weight bands, but rather a broad range of molecular weight with the average being considerably lower than that of the first three limed ossein extracts.

The acid pigskin gelatins contain no specific, sharp molecular weight fractions, the GPC profiles being quite broad and unimodal. AP-1 and AP-2 have the highest molecular weights while AP-3 and AP-4 appear to be approximately the same as each other but lower than either AP-1 or AP-2, as indicated also by intrinsic viscosity data. Samples AP-3, AP-4 and LO-4 give very similar molecular weight distribution profiles, which is probably due to the severity of the pretreatment and extraction processes, resulting in all labile bonds being hydrolyzed.

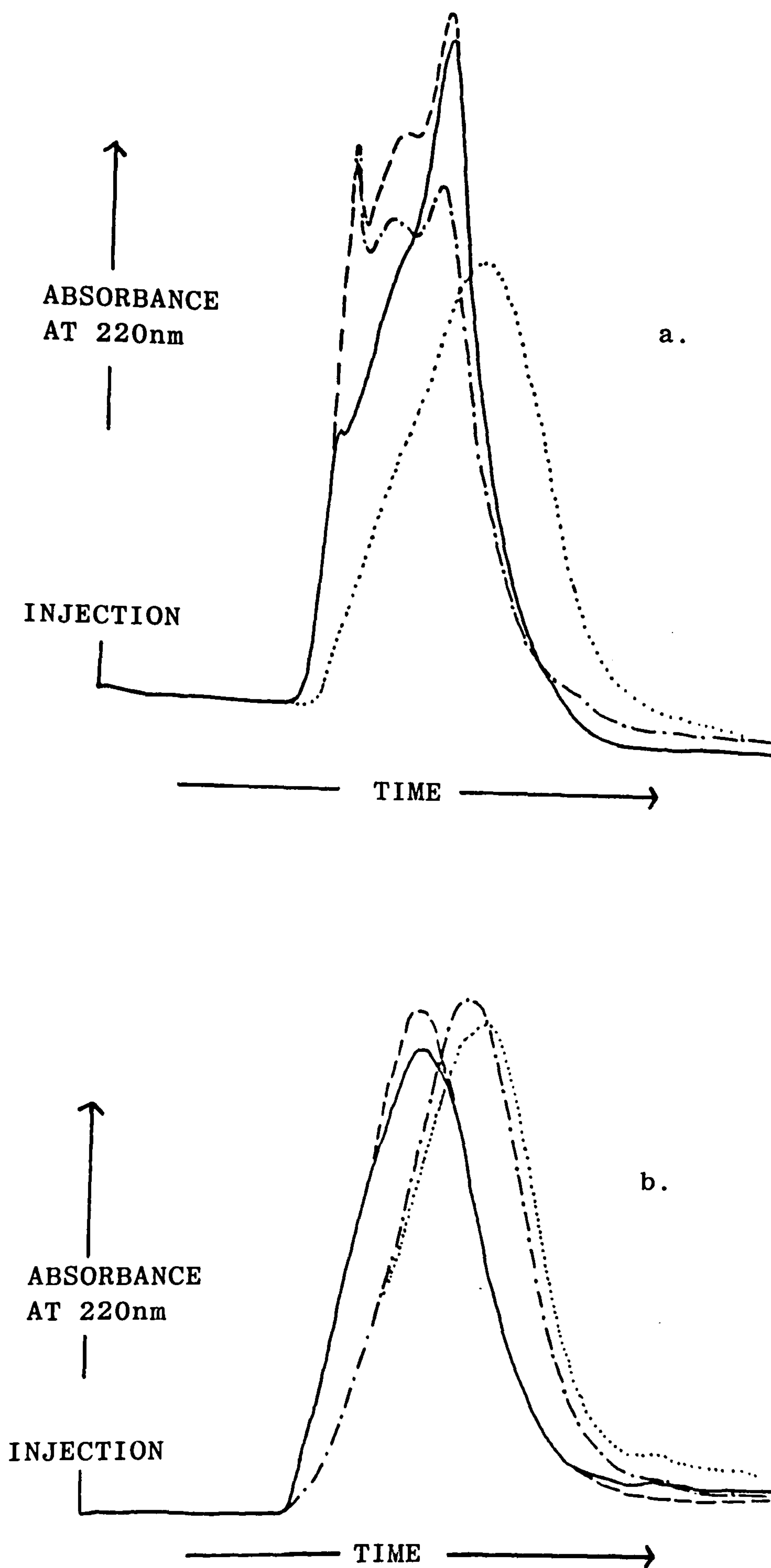


Figure 4.3. Gel permeation chromatography on the eight gelatin samples using Toyopearl HW 55S as the separating medium. (See text for other experimental details).  
 a) The limed ossein gelatins possessing large amounts of relatively high molecular weight material LO-1 (—), LO-2 (---), LO-3 (-.-.-), LO-4 (.....).  
 b) The acid pigskin gelatins AP-1 (—), AP-2 (---), AP-3 (-.-.-), AP-4 (.....).



### Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is of great use in characterising biopolymers. The basic principle behind electrophoresis is to observe the movement of the sample under the influence of an electric field, and thus separate the sample into its different constituents. In PAGE a large voltage is applied across a gel formed from the polymerisation of acrylamide, the concentration of acrylamide determining the strength of the gel and also, as a result of the steric effects of the gel pores, the rate at which proteins can move across the gel. Two other parameters influence the rate of movement, the charge on the protein and the electric field. The electric field is predetermined and set at the start of the experiment while the effect of the charge on the protein can be better defined by carrying out the experiment in the presence of sodium dodecyl sulphate (SDS) under reducing conditions. These conditions cause the protein molecules to assume a uniform extended rod shape with approximately the same charge per unit length. Under such circumstances, for a given acrylamide concentration, the rate of migration of the protein across the gel is dependent only on its molecular size and therefore separation according to molecular weight is possible.

The PAGE apparatus used to characterise the eight gelatin samples consisted of a Shandon Vertical Slab unit 2000 connected to an LKB Bromma power supply.

Due to the high polydispersity of the sample, a single concentration of acrylamide could not be used and three different concentrations (12%, 8% and 4%) were stacked on top of each other to produce a gradient of decreasing pore size on travelling through the gel. The polyacrylamide gels were prepared using the required amount of 30% acrylamide, 0.3 ml of 10% SDS, and a small amount of 10% ammonium persulphate ( $\sim 100\mu\text{l}$ ) and N,N,N',N'-tetramethylethylenediamine ( $\sim 20\mu\text{l}$ ) as activating agents. Once formed, approximately  $30\mu\text{l}$  of a 0.2% solution of each gelatin sample, under reducing conditions, was introduced into the wells formed in the stacking gel. The system was then set to run overnight and the gel stained (Coomassie blue) and destained (water/acetic acid/methanol mixture) the next day to reveal the protein bands as illustrated in Fig. 4.4.

Samples LO-1, LO-2 and LO-3 show the same characteristic bands with the amount of higher molecular weight material (bands closer to the top of the gel) increasing with extract number as also indicated by GPC. LO-4, however, has much less higher molecular weight material present and appears to be more polydisperse. The results for the acid pigskin samples are somewhat different to the limed ossein samples in that they show a more banded pattern. AP-1 and AP-2 clearly contain the highest molecular weight material, having bands in similar places to the limed ossein samples, whereas AP-3 and AP-4 are deficient in these bands, with a considerably higher fraction of lower molecular weight material.



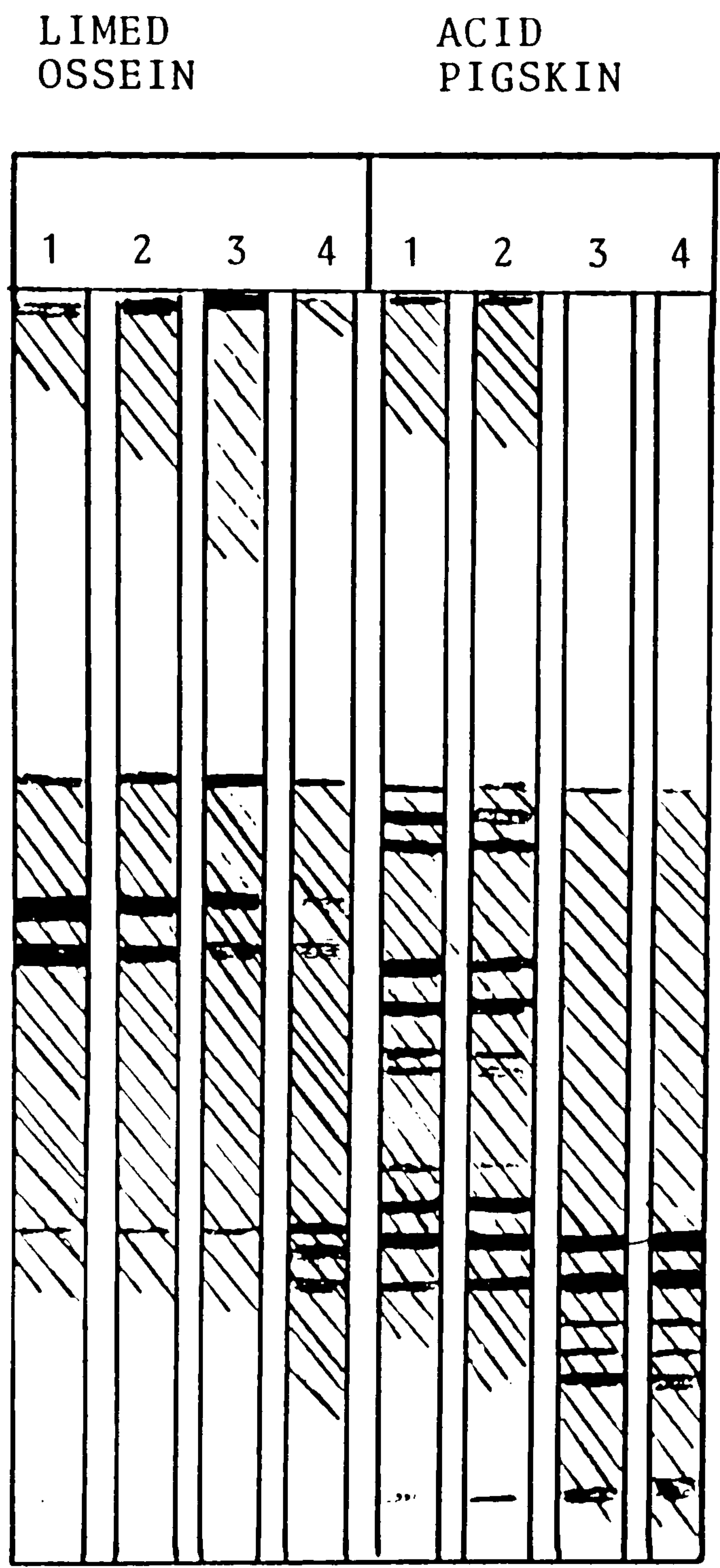


Figure 4.4. Polyacrylamide gel electrophoresis on the eight gelatin samples (see text for experimental details). The bands closer to the top represent the higher molecular weight species with lower molecular weight fragments travelling further through the gel.



To summarise therefore, it appears that the first three extracts of limed ossein gelatin are similar in terms of molecular weight, as are the first two extracts from acid pigskin gelatin. LO-4, AP-3 and AP-4, however, appear to have been substantially degraded.

## CHAPTER 5: MELTING BEHAVIOUR OF GELATIN GELS

### 5.1. INTRODUCTION

It is well known in the gelatin industry that the thermal history of gelatin gels can have a profound effect on their physical properties (Stainsby, 1977b; Ledward, 1986). Numerous studies on the melting of gelatin gels and collagen by various physico-chemical techniques have been reported (Beier and Engel, 1966; Privalov and Tiktopulo, 1970; Petrie and Becker, 1970; Godard *et al.*, 1978; Djabourov and Papon, 1983) but the results are often conflicting and vary according to the sample under test and the specific experimental conditions.

One of the most interesting aspects of the influence of thermal history is the increased rigidity of gels formed by holding a sample for a period of time at a moderate temperature prior to quenching to the final temperature, over gels quenched directly to the lower temperature (te Nijenhuis, 1981a,b). The final value of the rigidity modulus depends on the temperature of pre-maturation and the length of time held at this temperature. Te Nijenhuis (1981b) suggested that at any given temperature certain structures are stable, and that the number of these structures continues to increase with time, at the same rate, irrespective of

temperature, provided that the temperature is lower than that required to form the structure. This explanation, however, does not explain the observed effect of pre-maturation on the final value of the rigidity modulus. This chapter reports a detailed re-examination of this essentially unexplained behaviour, using a variety of different techniques to monitor the melting behaviour of gels formed under various curing regimes.

## 5.2. MELTING OF HELICAL STRUCTURE AS MONITORED BY DIFFERENTIAL SCANNING CALORIMETRY AND OPTICAL ROTATION

Fig. 5.1a shows the time-course of helix structure regeneration (as monitored by optical rotation at  $\lambda = 365 \text{ nm}$ ) for a  $20 \text{ mg ml}^{-1}$  LO-1 solution, after rapid quenching to  $5^\circ\text{C}$  from  $45^\circ\text{C}$ . The initial rapid change in optical rotation is followed by a slower logarithmic stage and, after approximately 12 hours, the overall helix fraction changes only very slightly with time. Melting endotherms for the same solution gelled at  $5^\circ\text{C}$  for 16 hours, obtained by differential scanning calorimetry (DSC), at various scan rates, are shown in Fig. 5.1b. The increase in scan rate has little, if any, effect on the shape of the melting endotherm and merely shifts its position to higher temperatures; this can be attributed to thermal lag within the calorimeter. A plot of transition midpoint temperature ( $T_m$ ) versus scan rate (Fig. 5.1c)



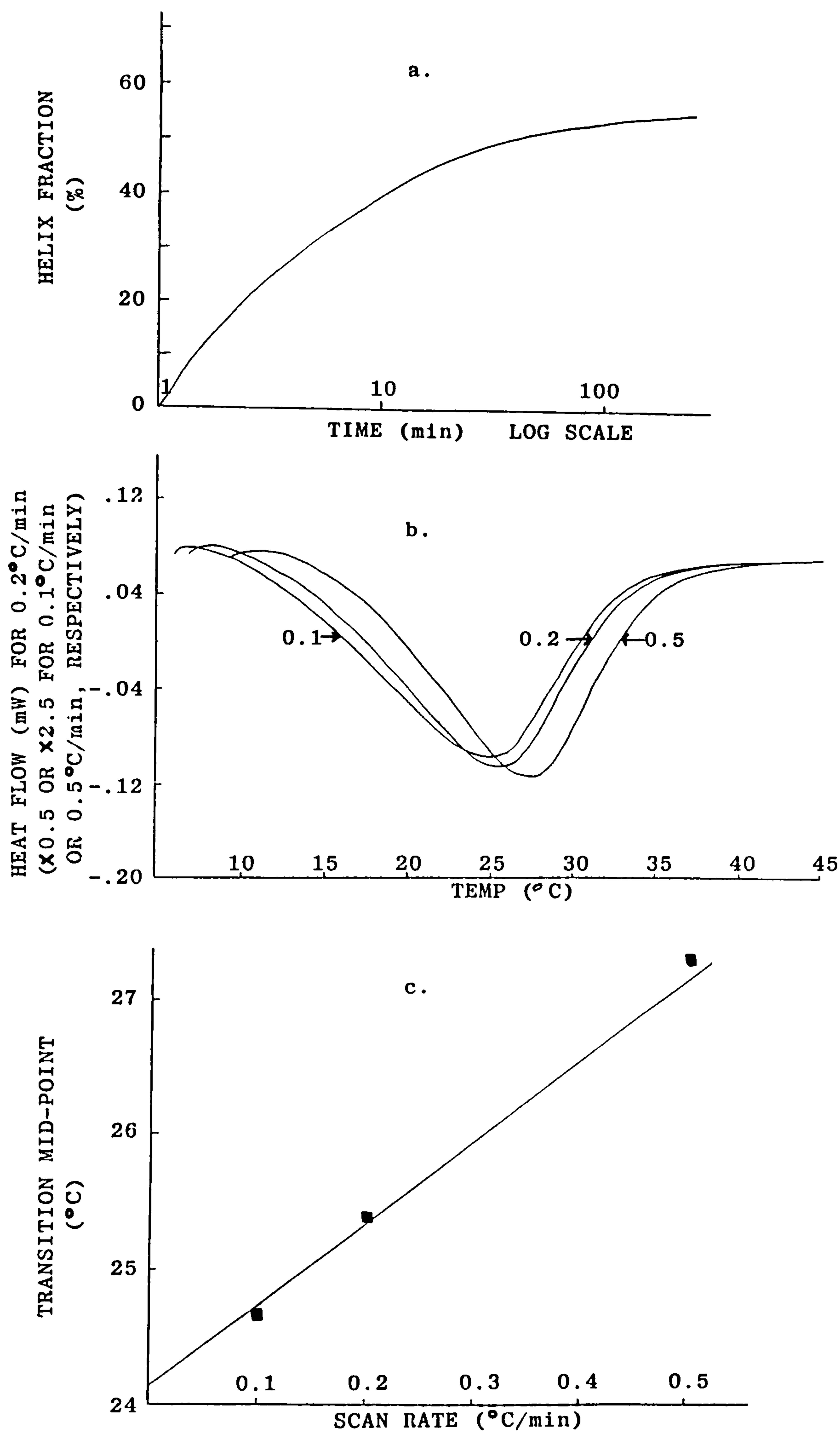


Figure 5.1. a) Regeneration of ordered helical structure (monitored by optical rotation) as a function of time on quenching a solution of LO-1 ( $20 \text{ mg ml}^{-1}$ ) to  $5^\circ\text{C}$ ;  
 b) DSC melting endotherms of the LO-1 solution at various scan rates ( $0.1$ ,  $0.2$  and  $0.5^\circ\text{C min}^{-1}$ ) after being held at  $5^\circ\text{C}$  for 16 hours;  
 c) Effect of increase in scan rate on the apparent position of the transition mid-point temperature of the LO-1 solution.

is linear and, by extrapolation, can be used to obtain the values of  $T_m$  at zero scan rate. For the scan rate of  $0.1^\circ\text{C min}^{-1}$  used in most of the work reported in this thesis, the deviation from the extrapolated value is less than  $1^\circ\text{C}$ .

The physical properties of gelatin gels, as mentioned previously, are crucially dependent on their thermal history. This has been investigated systematically using DSC to observe the melting behaviour of gels held at different temperatures for different lengths of time. LO-1 ( $20\text{ mg ml}^{-1}$ ) gels were formed by quenching the sample from  $45^\circ\text{C}$  to the temperature under investigation and holding at that temperature for varying lengths of time before further cooling to  $5^\circ\text{C}$  for 16 hours and melting out. 16 hours at  $5^\circ\text{C}$  was chosen as a reference state for all scans because, after this time, further conformational ordering as judged by optical rotation (Fig. 5.1a) was negligible over the period of the DSC measurement ( $\sim 7$  hours).

The effect on the melting endotherms of pre-maturation at higher temperatures is illustrated in Figs. 5.2a, b, c, d for holding temperatures of 10, 15, 20, and  $25^\circ\text{C}$  respectively. For any particular holding temperature ( $T_o$ ) a family of curves is obtained, showing progressive development of a higher-melting peak with increased length of time at that holding temperature. The thermograms assume a distinct bimodal character with the

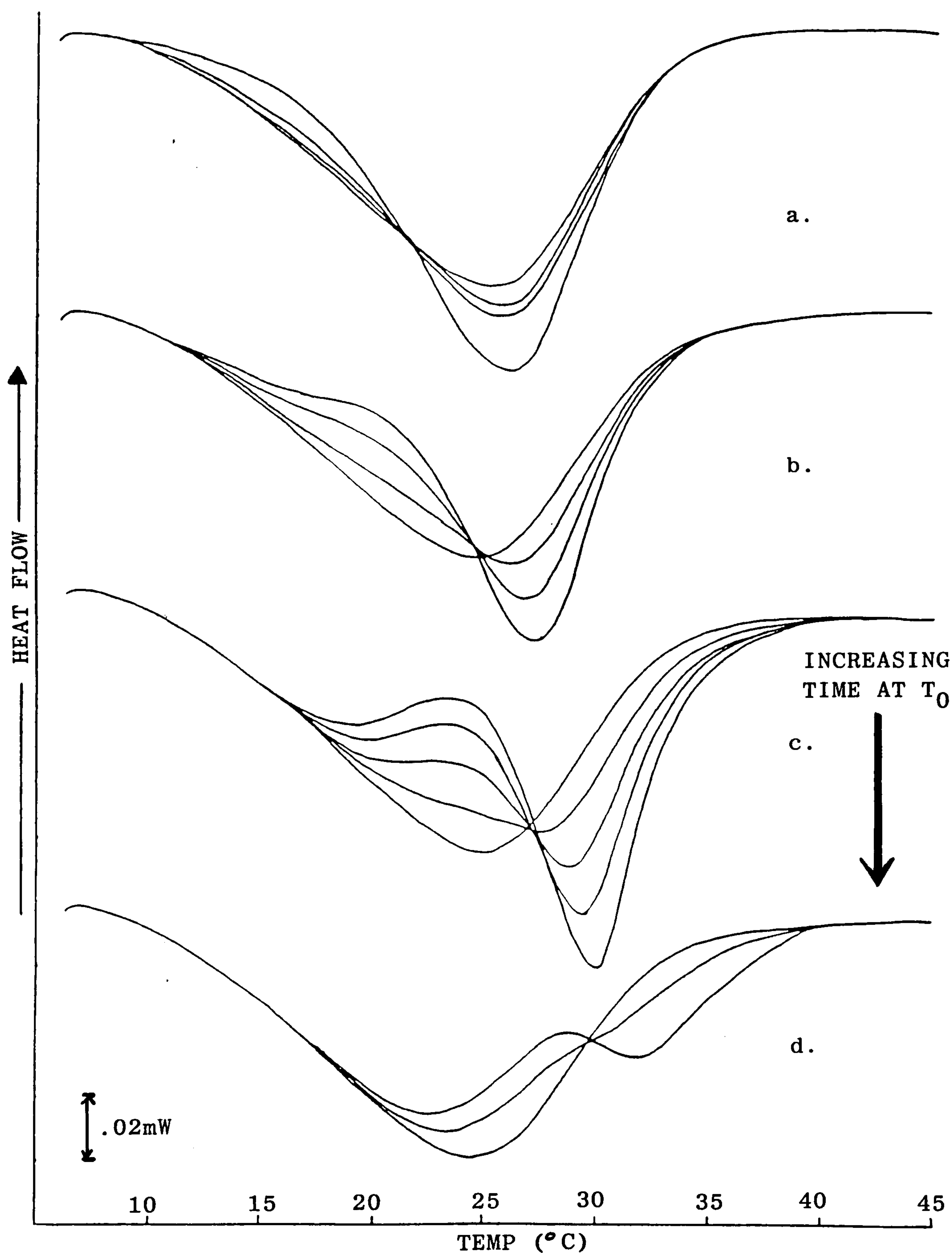


Figure 5.2. DSC melting endotherms for LO-1 gels ( $20 \text{ mg ml}^{-1}$ ) held at various temperatures ( $T_0$ ) for various lengths of time, prior to final quenching to  $5^\circ\text{C}$  for 16 hours:

- a) Sample held at  $10^\circ\text{C}$  for 0, 1.5, 4 and 42 hours.
- b) Sample held at  $15^\circ\text{C}$  for 0, 1.5, 7 and 24 hours.
- c) Sample held at  $20^\circ\text{C}$  for 0, 2, 7, 24 and 72 hours.
- d) Sample held at  $25^\circ\text{C}$  for 0, 8 and 35 hours.



families of curves, for each holding temperature, crossing at a single isobestic point, and the position of the higher-melting peak increasing with increasing  $T_0$ . In all cases, however, the overall enthalpy change (peak area) is essentially constant (Table 5.1) at approximately  $3.3 \text{ kJ mol}^{-1}$  (assuming an average residue molecular weight of 100) and the net helix fraction is also virtually unchanged (at  $\sim 60\%$ ).

Generation of a population of higher-melting species has also been conveniently observed by monitoring the temperature course of helix melting using optical rotation as an index of helix fraction. Fig. 5.3 illustrates that the decrease in helix fraction with incremental increase in temperature ( $da/dT$ ) is closely superimposable on the overall melting profile obtained by DSC, both for samples quenched directly to  $5^\circ\text{C}$  and for those held for a time at higher temperatures to give bimodal melting behaviour.

Fig. 5.3 also shows the temperature-course of helix melting for the same sample when it is held for the same length of time at  $T_0$ , but then melted directly from this temperature without first cooling to  $5^\circ\text{C}$ . Under these conditions, the temperature-dependence of  $da/dT$  is unimodal, joins the bimodal melting curve at the isobestic point, and follows the same temperature-course thereafter. Melting endotherms obtained by DSC on samples melted directly from the holding temperature

similarly follow the same profile as the higher melting peak of samples in which the standard 16 hours at 5°C was utilised as can be seen for the holding temperatures of 5, 15, 20 and 25°C in Fig. 5.4. It therefore appears that the bimodal melting behaviour, characterised independently by DSC and optical rotation, has its origin in the selective formation at  $T_0$  of a population of helices of greater thermal stability than those formed at lower temperature (5°C), which then remain virtually unchanged when the temperature is subsequently decreased.

The close superposition (Fig. 5.3) of the temperature-course of melting by the techniques of DSC and optical rotation argues against stabilisation of helices by helix-helix aggregation as has been proposed in the past (Godard *et al.*, 1978; Tomka *et al.*, 1975). Only if the enthalpy change associated with aggregation was extremely small and therefore undetectable by DSC, could aggregation of helices be a possible stabilizing force; a measurable aggregation enthalpy would alter the melting profile obtained by DSC compared to that obtained by optical rotation, which reflects only the destruction of helical structure.

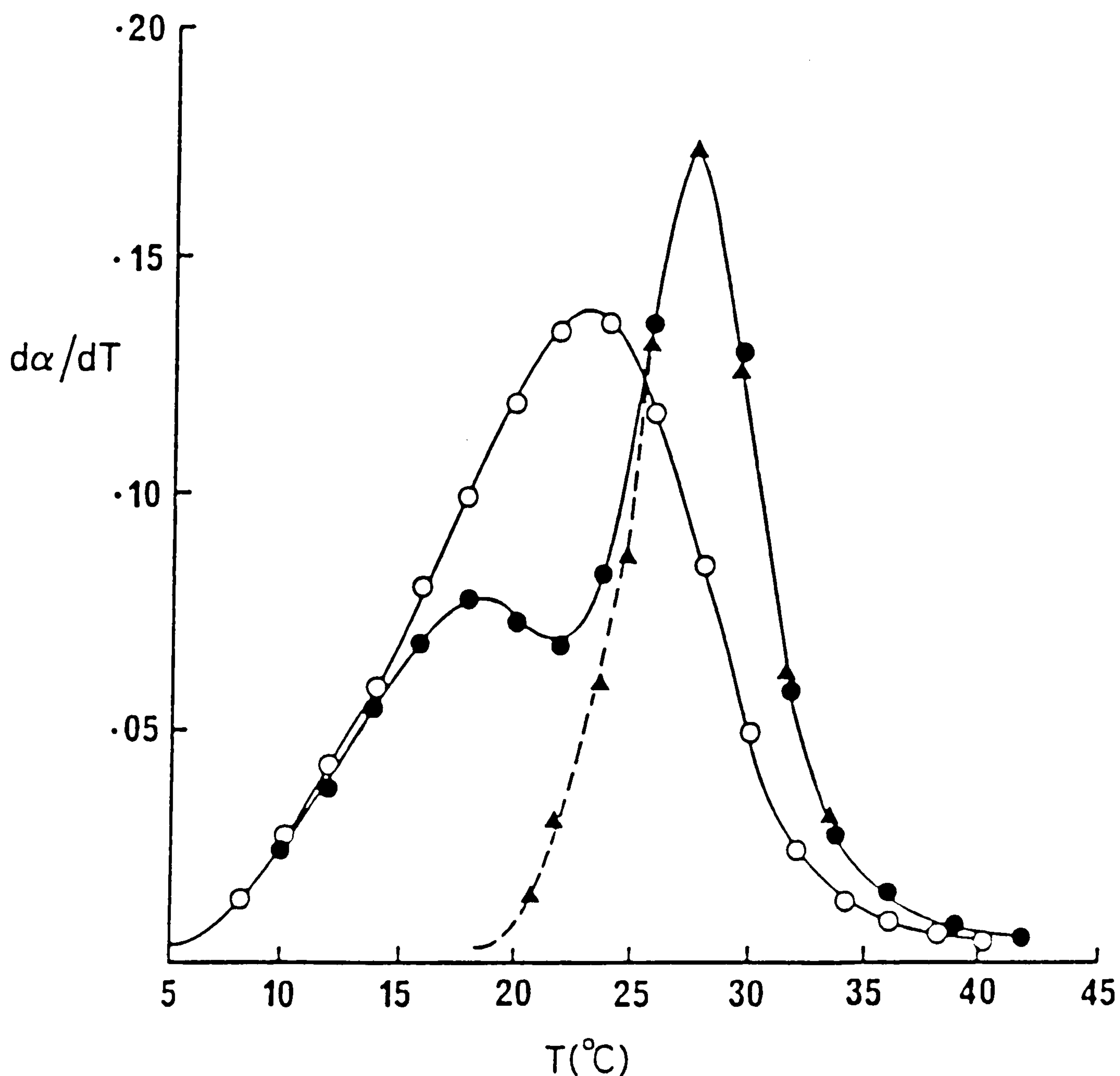


Figure 5.3. Thermal melting of LO-1 gels ( $20 \text{ mg ml}^{-1}$ ) quenched to  $5^\circ\text{C}$  for 16 hours directly ( $\circ$ ) or after holding for 24 hours at  $20^\circ\text{C}$  ( $\bullet$ ); symbols show local conformation change by optical rotation ( $d\alpha/dT$ ) and solid lines show overall enthalpy change (Fig. 5.2c) by DSC. Also shown ( $\blacktriangle$ ) is the optical rotation change for the same sample melted directly after holding at  $20^\circ\text{C}$  for 24 hours.



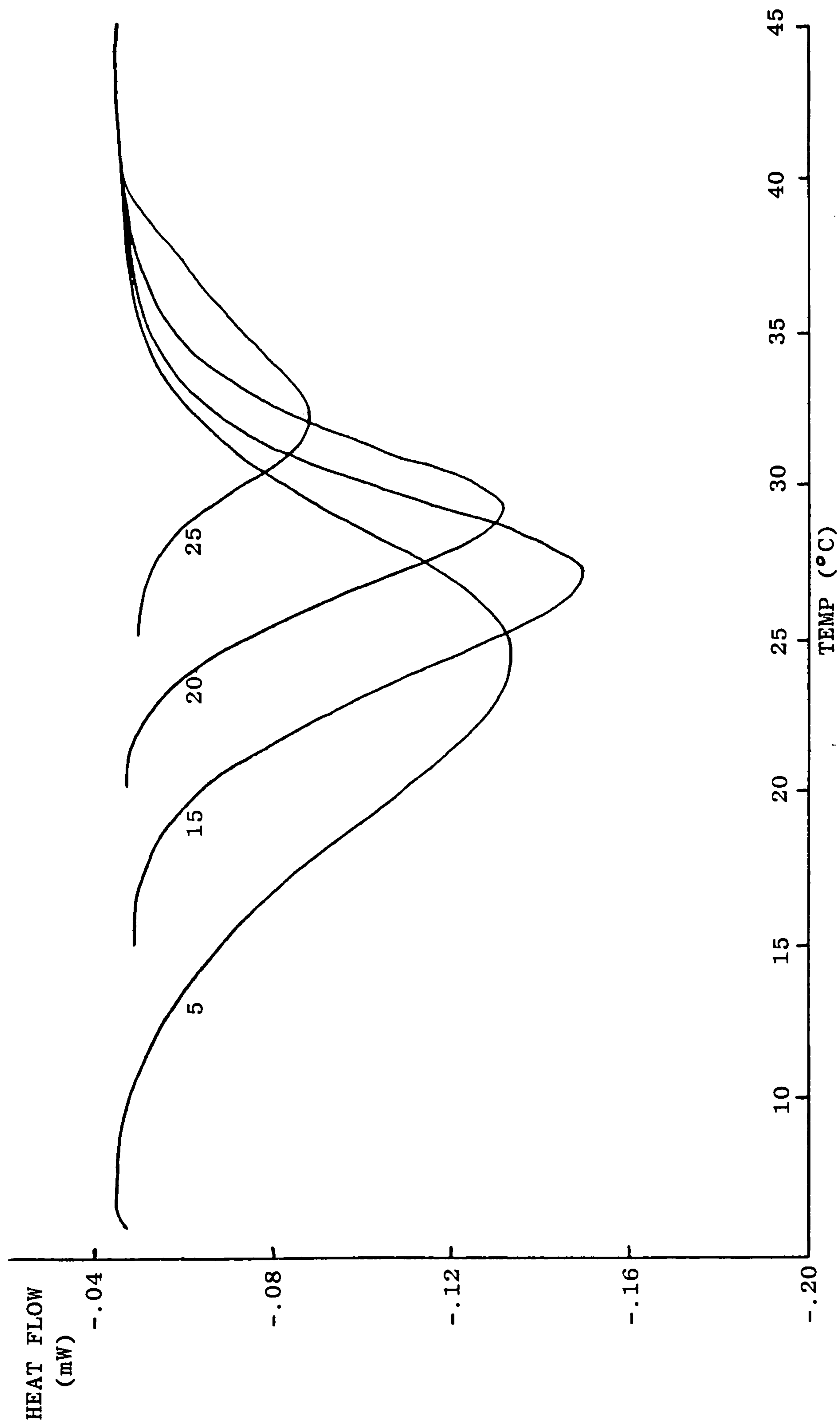


Figure 5.4. DSC melting endotherms of LO-1 gels ( $20 \text{ mg ml}^{-1}$ ) melted directly from the holding temperatures of 5, 15, 20 and  $25^\circ\text{C}$  after 16, 24, 17 and 40 hours respectively.

### 5.3. EFFECT OF PRE-MATURING AT HIGHER TEMPERATURES ON THE RIGIDITY MODULUS, $G'$

Holding a sample at higher temperatures, prior to aging at 5°C, has little effect on the overall helix fraction measured by optical rotation or the enthalpy of melting obtained from DSC (see Table 5.1) compared to a sample cooled directly to 5°C. There is, however, a significant effect on the rigidity ( $G'$ ) of the gel, which has been reported by various workers (te Nijenhuis, 1981a,b; Stainsby, 1962, 1969).

To investigate this further, 20 mg ml<sup>-1</sup> LO-1 samples were subjected to the same temperature regime used in the DSC measurements and the loss of rigidity on melting monitored. The results for a sample held at 20°C for 24 hours and then cooled to 5°C for the standard 16 hours, are illustrated in Fig. 5.5a,b along with the results for a sample quenched directly to 5°C. The gel cured at the higher temperature has a marked increase in rigidity over the gel cured only at 5°C (830 Pa compared to 630 Pa), with the value remaining higher throughout the melting process. Furthermore, in the case of samples held for sufficiently long to give obvious biphasic melting behaviour by DSC, the temperature-course of loss of rigidity on heating ( $dG'/dT$ ) also shows bimodal character (Fig. 5.5b).

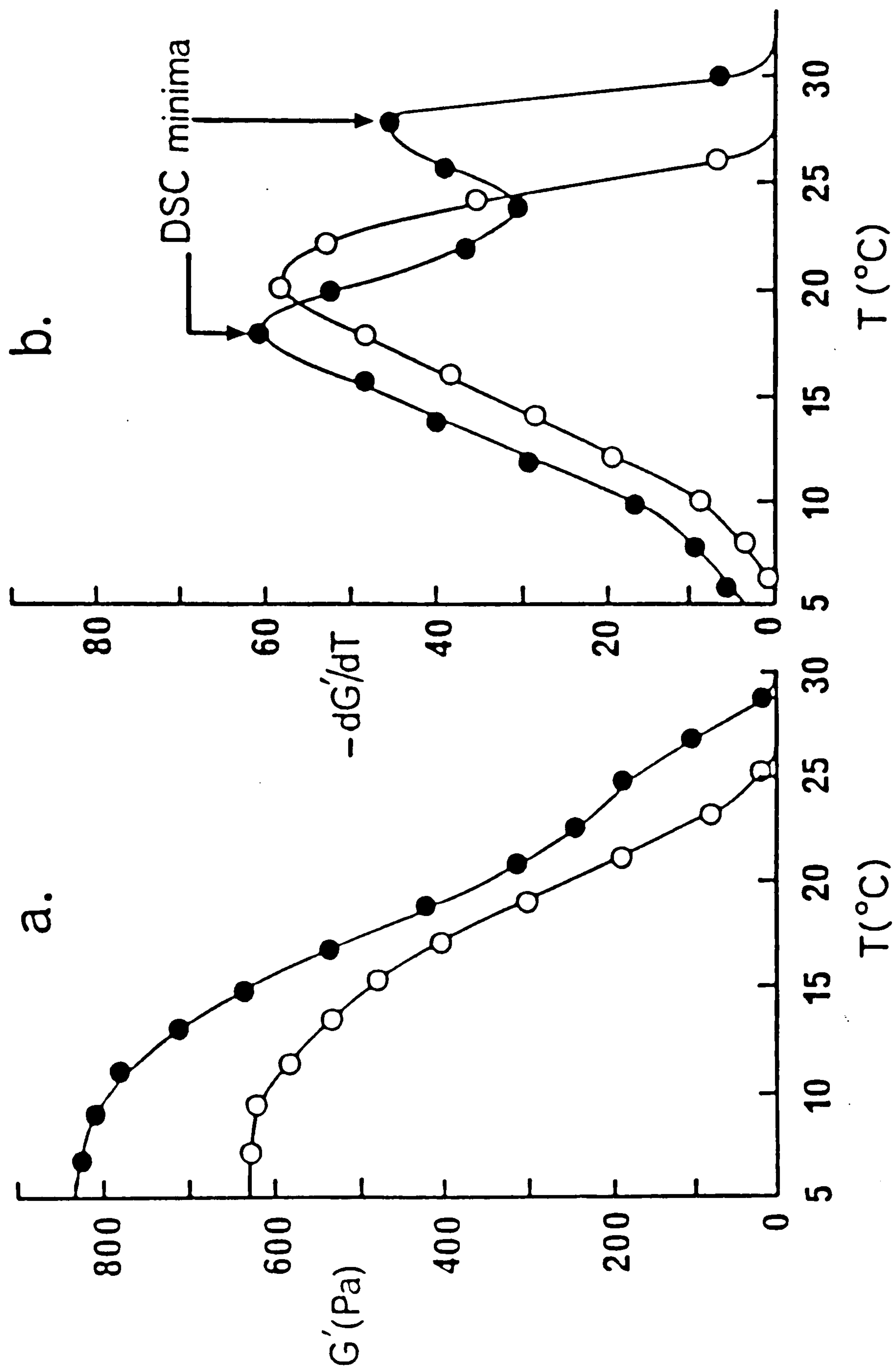


Figure 5.5. Temperature-dependence of rigidity ( $G'$ , 0.5 Hz) for LO-1 gels ( $20 \text{ mg ml}^{-1}$ ) quenched to  $5^{\circ}\text{C}$  for 16 hours, either directly ( $\circ$ ) or after holding for 24 hours at  $20^{\circ}\text{C}$  ( $\bullet$ ); the final helix fraction is virtually identical in both cases ( $f_H \approx 0.6$ ): a) absolute values of  $G'$  and b) rate of change of  $G'$  with increasing temperature ( $dG'/dT$ ).



The shapes of the  $dG'/dT$  melting profiles are not superimposable upon those obtained by optical rotation and DSC (Fig. 5.3). This is not a surprising result as DSC and optical rotation monitor the melting of helices at a molecular level, whereas the rigidity of a gel is a measure of the long range network properties. The positions of the two peaks, however, correspond closely for all three physical techniques, indicating the techniques are merely monitoring different resulting aspects of the same underlying process.

#### 5.4. STABILITY AT DIFFERENT TEMPERATURES

The melting profiles, obtained from both DSC and OR, of samples held for long periods of time at different holding temperatures and subsequently melted directly from these temperatures, are shown in Fig. 5.4 and Fig. 5.6, respectively. The melting points ( $T_m$ ) for any particular  $T_o$  are approximately the same by both techniques and are tabulated in Table 5.2, which lists the temperature when half of the melting transition has occurred. It can be seen that increased holding temperature leads to increased thermal stability which has been reported previously by Beier and Engel (1966), working on renatured collagens. They, however, found biphasic melting behaviour at almost all holding temperatures, with holding temperatures above 15°C

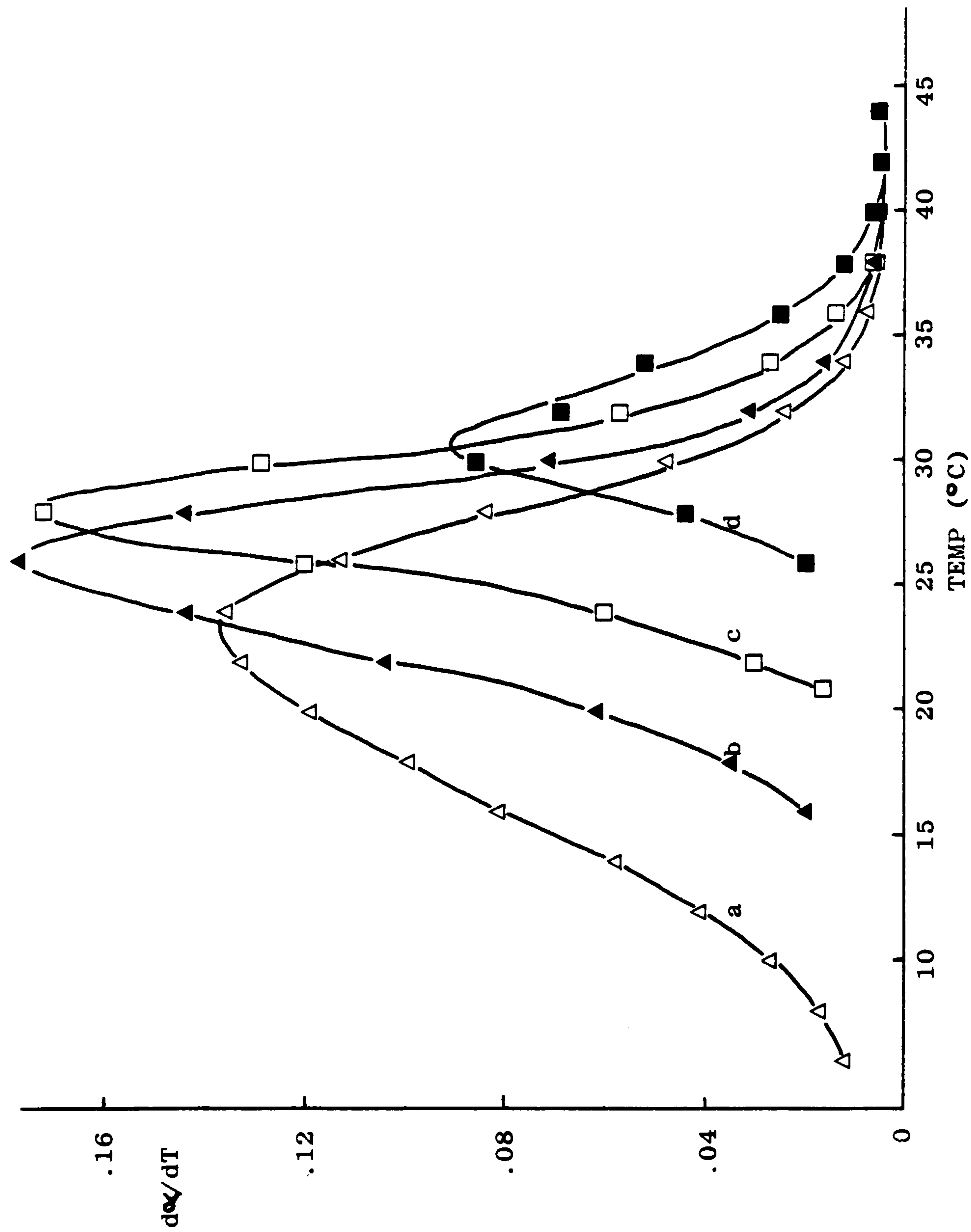


Figure 5.6. Thermal melting profiles showing local conformational change, monitored by optical rotation, for LO-1 gels (20 mg ml<sup>-1</sup>) held for: a) 16 hours at 5°C; b) 25 hours at 15°C; c) 39 hours at 20°C and d) 51 hours at 25°C.

Table 5.1. Enthalpies of melting for 20 mg ml<sup>-1</sup> LO-1 gels formed by quenching the sample to various temperatures (T<sub>0</sub>) for varying lengths of time prior to further cooling at 5°C for 16 hours.

T <sub>0</sub> (°C)	LENGTH OF TIME AT T <sub>0</sub> (HOURS)	ENTHALPY OF MELTING (J/g SOLUTION)	ENTHALPY OF MELTING <sup>a</sup> (kJ/mol)
5	16	0.652 ±0.02	3.26 ±0.1
10	1.5	0.627 "	3.13 "
10	4	0.626 "	3.13 "
10	42	0.648 "	3.24 "
15	1.5	0.682 "	3.40 "
15	7	0.683 "	3.42 "
15	24	0.688 "	3.44 "
20	2	0.678 "	3.39 "
20	7	0.684 "	3.42 "
20	24	0.687 "	3.43 "
20	72	0.701 "	3.50 "
25	8	0.656 "	3.28 "
25	35	0.693 "	3.46 "

a Calculated assuming an average amino acid residue molecular weight of 100 for gelatin.

Table 5.2. Melting points (T<sub>m</sub>) of 20 mg ml<sup>-1</sup> LO-1 gels cured for a long period of time at holding temperatures (T<sub>0</sub>). The value of T<sub>m</sub> is the temperature at which half of the transition has occurred as judged by optical rotation.

T <sub>0</sub> (°C)	T <sub>m</sub> (°C)
5	22.0
10	23.5
15	25.5
20	28.1
25	31.1



giving rise to a specific melting peak at the native collagen melting temperature. No such peak was ever observed in the work reported in this thesis. The relationship between  $T_m$  and  $T_o$  will be discussed in further detail later (Chapter 7 ).

Previous investigators (Eldridge and Ferry, 1954; Stainsby, 1977b) have reported the annealing in of more stable structures with increased time at a particular holding temperature. According to the results presented in Fig. 5.2, for the LO-1 sample used throughout this work, however, this does not appear to be the situation. Instead, for any specific  $T_o$ , there is an approximately Gaussian population of stable structures centred around the corresponding  $T_m$ , which begins to form on reaching the quench temperature and progressively increases in magnitude with no change in  $T_m$ . Only in the case of the sample quenched directly to 5°C is there any evidence of a significant annealing effect where, after 90 hours at 5°C, the shape of the melting endotherm becomes less broad, presumably as a result of the conversion of the least stable structures into ones possessing higher thermal stability (see Fig. 5.7).

It is well known (Ferry, 1948a) that raising the temperature of a gelatin gel after pre-cooling at a lower temperature, leads to rapid attainment of the rigidity value which would have been obtained by curing for long periods of time at the higher temperature only;

a similar effect is also observed with optical rotation measurements. This behaviour has frequently been utilised as a method for obtaining a so-called 'equilibrium gel' at high temperatures, avoiding the long aging period required if pre-cooling was not used. The situation is not as simple as this, however, as indicated in Fig. 5.8 which illustrates the melting endotherms of gels cured first at 5°C, then raised to 25°C for varying lengths of time, followed by a further 16 hours at 5°C. The position of the higher-melting peak increases with increasing time at 25°C, approaching, but not reaching, the peak position of samples cured directly at 25°C without the pre-cooling period at 5°C (Fig. 5.2d). The size of the second peak, however, does not vary greatly with increased time at 25°C, indicating that the helix fraction is approximately constant even though the stability of the structures is still changing with time. Thus, although pre-cooling may very quickly lead to an 'equilibrium helix fraction' on subsequent heating, the stability of the helices continues to change over a long period of time.

#### 5.5. NATURE OF THE OBSERVED INCREASED STABILITY

As shown above, the triple helical regions within a gelatin gel have varying thermal stabilities which, with increasing renaturation temperature, approach but never reach, that of native collagen ( $T_m$  36°C - 40°C). These

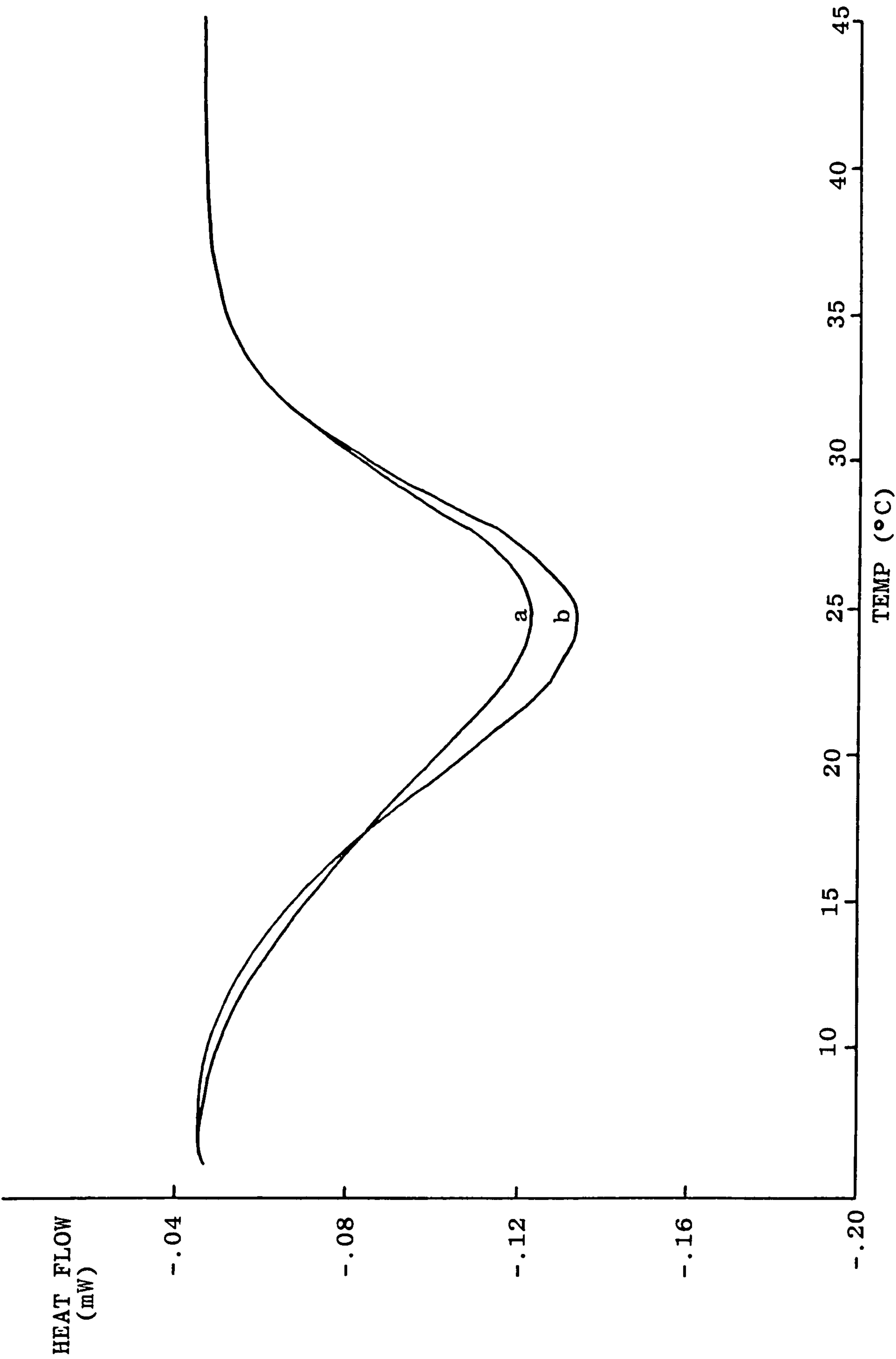


Figure 5.7. Comparison of the DSC melting endotherms obtained on melting an LO-1 gel (20 mg ml<sup>-1</sup>) cured for 16 hours at 5°C (a) and 90 hours at 5°C (b). (The melting profile becomes slightly narrower with an excessive length of time at 5°C).



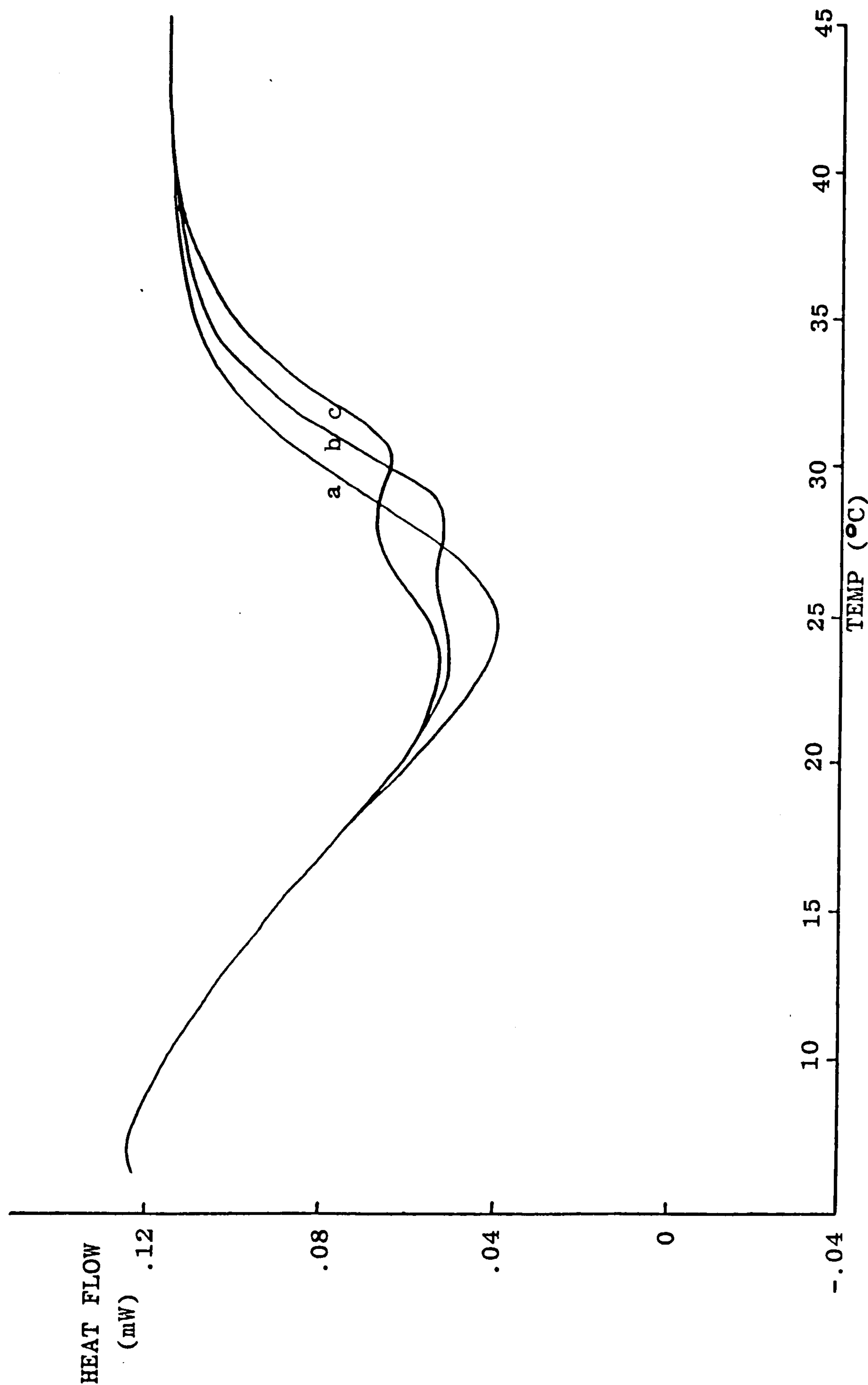


Figure 5.8. DSC melting endotherms of LO-1 gels ( $20 \text{ mg ml}^{-1}$ ) which have been subjected to curing regimes involving 16 hours at  $5^\circ\text{C}$  followed by a period of time at  $25^\circ\text{C}$  before being further quenched to  $5^\circ\text{C}$  for 16 hours.  
a) zero time at  $25^\circ\text{C}$   
b) 1 hour at  $25^\circ\text{C}$   
c) 7 hours at  $25^\circ\text{C}$ .

results obviously raise the question: What is the molecular basis for the formation of a population of helices of greater stability by curing at higher temperatures?

#### 5.5.1. Specific interactions

One possible answer to the question posed above could be that, at low temperatures, helices of low stability are formed by a non-specific triple helical interaction of the gelatin chains, whereas, at higher temperatures, annealing of the helical structures can occur, resulting in more specific, thermodynamically favourable interactions between the amino acid residues in the gelatin chains (for example helical segments containing one  $\alpha_2$  chain and two  $\alpha_1$  chains in correct register). This possibility has been investigated using the technique of circular dichroism (CD) which, as stated in Chapter 3, is particularly sensitive to detail of macromolecular organisation and, as such, might be expected to detect any differences in local molecular environments caused by specific monomer residue interactions.

CD studies

The CD spectra ( $\lambda = 250-210$  nm) obtained on melting a 20 mg ml<sup>-1</sup> gelatin sample, after curing for 16 hours at 5°C only and for 24 hours at 20°C followed by 16 hours at 5°C, are shown in Figs. 5.9a and 5.9b respectively. For samples in the gel state there is a positive peak at 221 nm; this peak has been reported previously by other workers studying collagen (Nishio and Hayashi, 1985) and is therefore further confirmation that the gelatin molecules are undergoing some partial reversion to the collagen triple helix conformation. There are no appreciable differences in the CD spectra of the gels cured under the two cooling regimes which, as with the optical rotation results, indicates that the net helix fractions within the two gels are the same.

On melting out (see Figs. 5.9a and b), the peak at 221 nm decreases in size as the conformation changes from triple helical to random coil. For the sample previously cured at high temperature, the disappearance of the peak on melting occurs at higher temperatures than for the sample cured only at 5°C and, as expected, a plot of the temperature-course of loss of peak height versus temperature is bimodal (Fig. 5.10). For the sample pre-matured at 20°C prior to aging at 5°C, Fig. 5.11 shows 'difference spectra' between the CD spectra obtained at 5°C and 22°C and the CD spectra obtained at 27°C and 39°C respectively. Clearly, there is no significant difference (within experimental error)



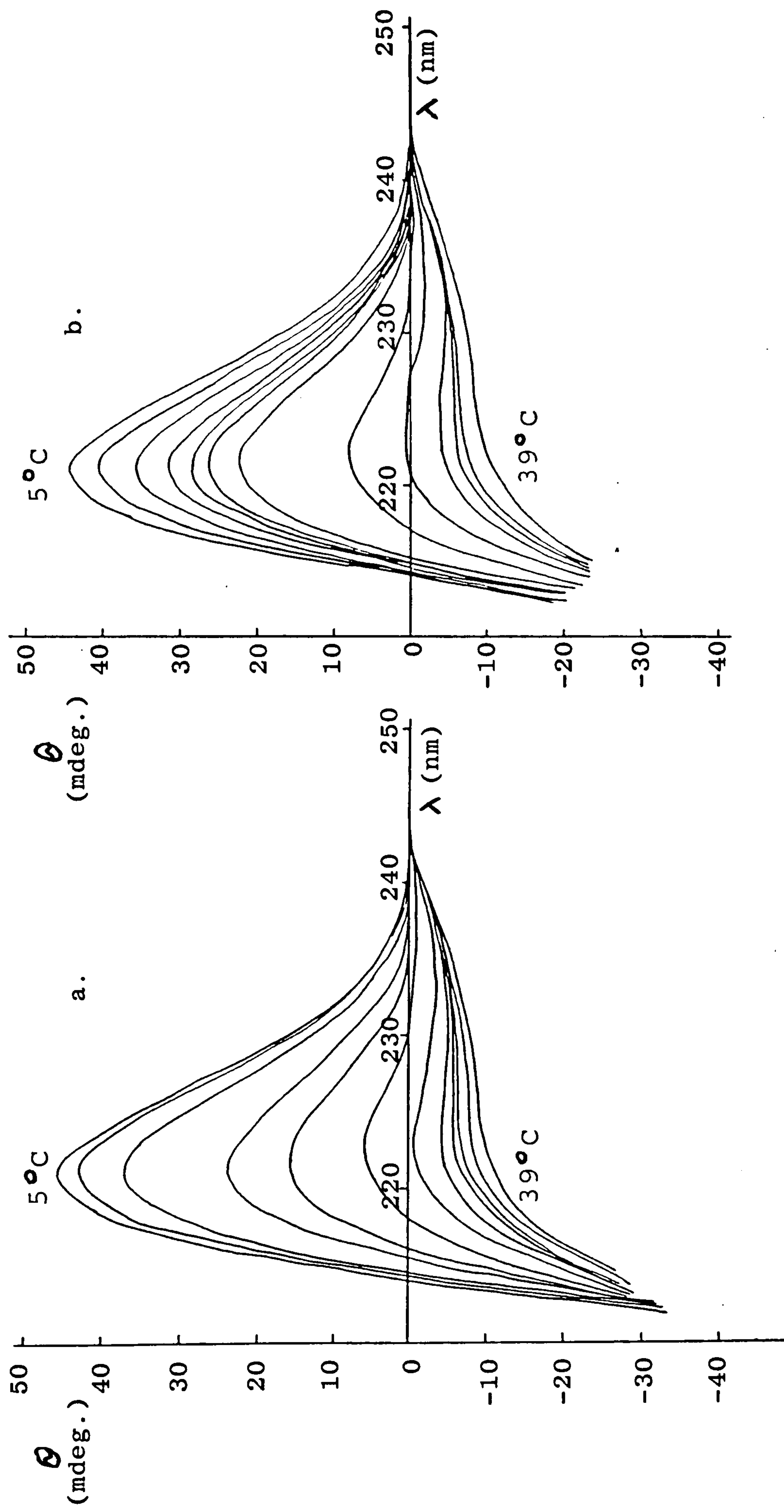


Figure 5.9. The CD spectra obtained on melting gels formed by quenching at 5°C for 16 hours (a) and at 20°C for 24 hours followed by 16 hours at 5°C (b). The curves illustrated are at temperatures of 5, 10, 15, 20, 22, 25, 27, 29, 31, 33, 35 and 39°C (a) and at temperatures of 5, 10, 15, 17, 19, 20, 22, 27, 29, 31, 33, 35 and 39°C (b). The cell path-length was 0.05 mm and the gelatin concentration was 20 mg ml<sup>-1</sup>.

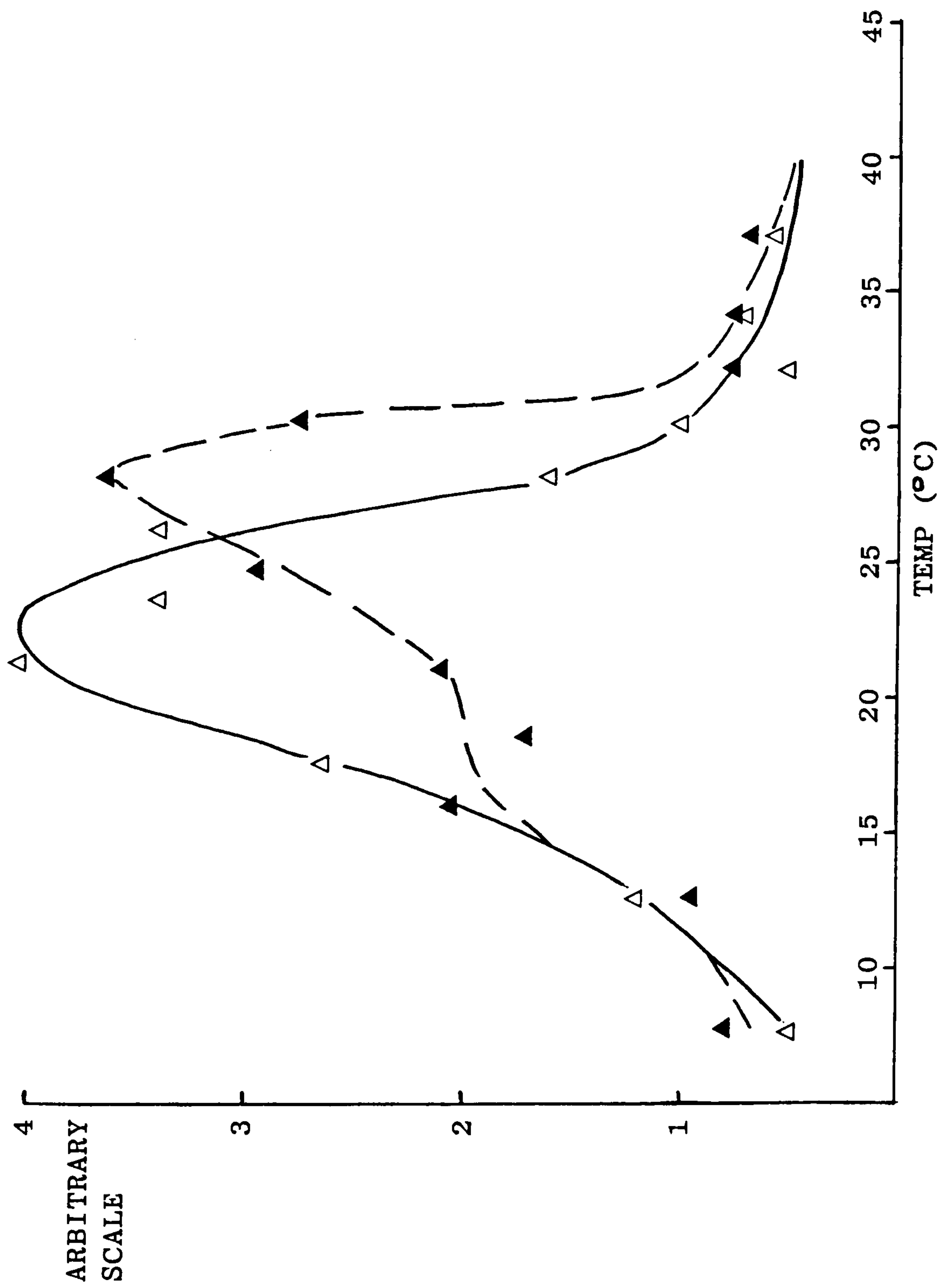


Figure 5.10. The decrease in CD peak height at 221 nm (arbitrary scale) as a function of temperature on melting an LO-1 gel ( $20 \text{ mg ml}^{-1}$ ) previously cured at  $20^\circ\text{C}$  for 24 hours followed by  $5^\circ\text{C}$  for 16 hours ( $\blacktriangle$ ). Evidence of biphasic melting is present. Also shown is the unimodal temperature course of loss of peak height for the sample quenched to  $5^\circ\text{C}$  directly ( $\Delta$ ).

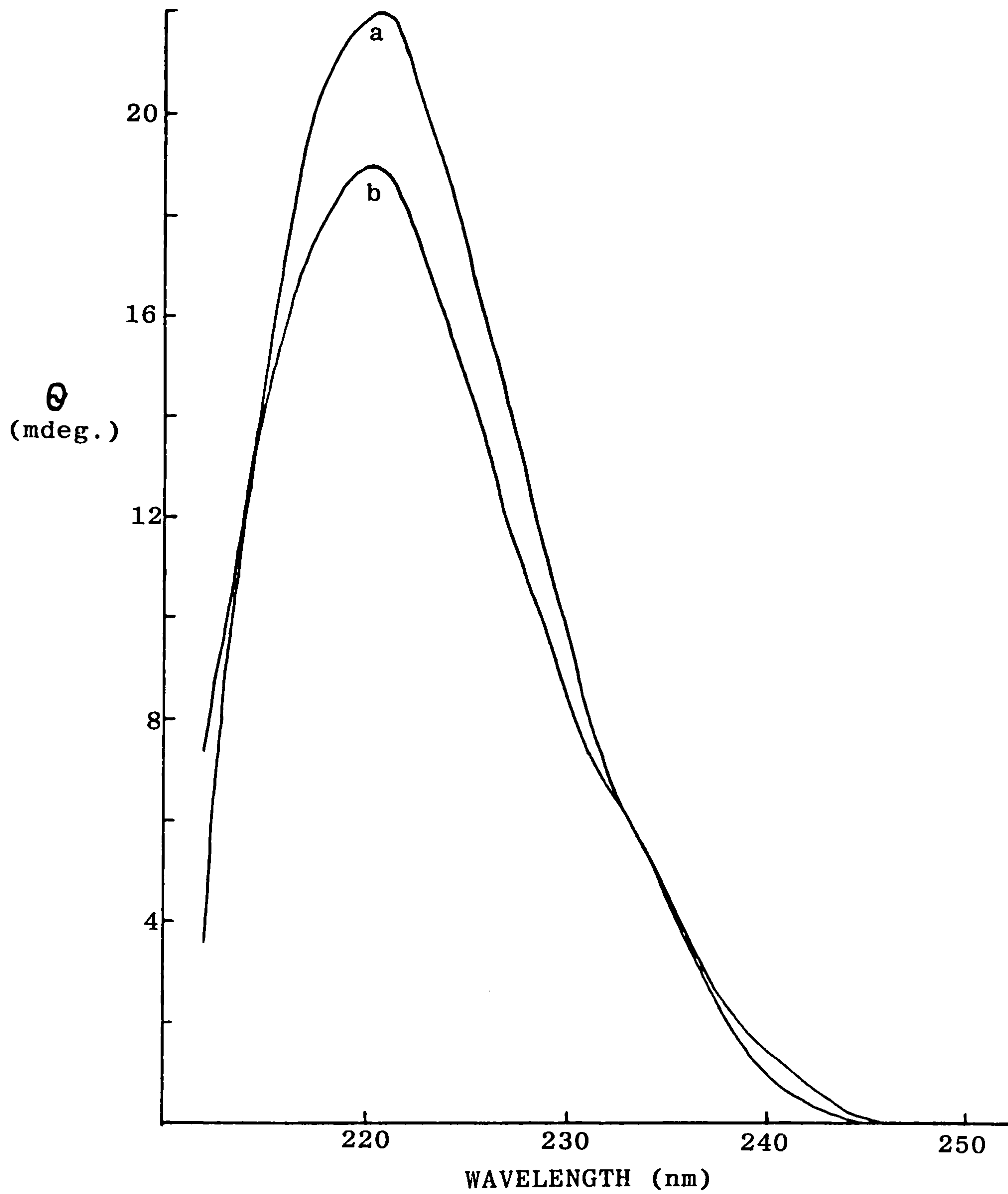


Figure 5.11. Comparison of CD 'difference spectra' obtained by subtraction of the CD spectra at 22°C from that at 5°C (a) and the CD spectra at 39°C from that at 27°C (b) for the gelatin whose CD behaviour is illustrated in Fig. 5.9b.



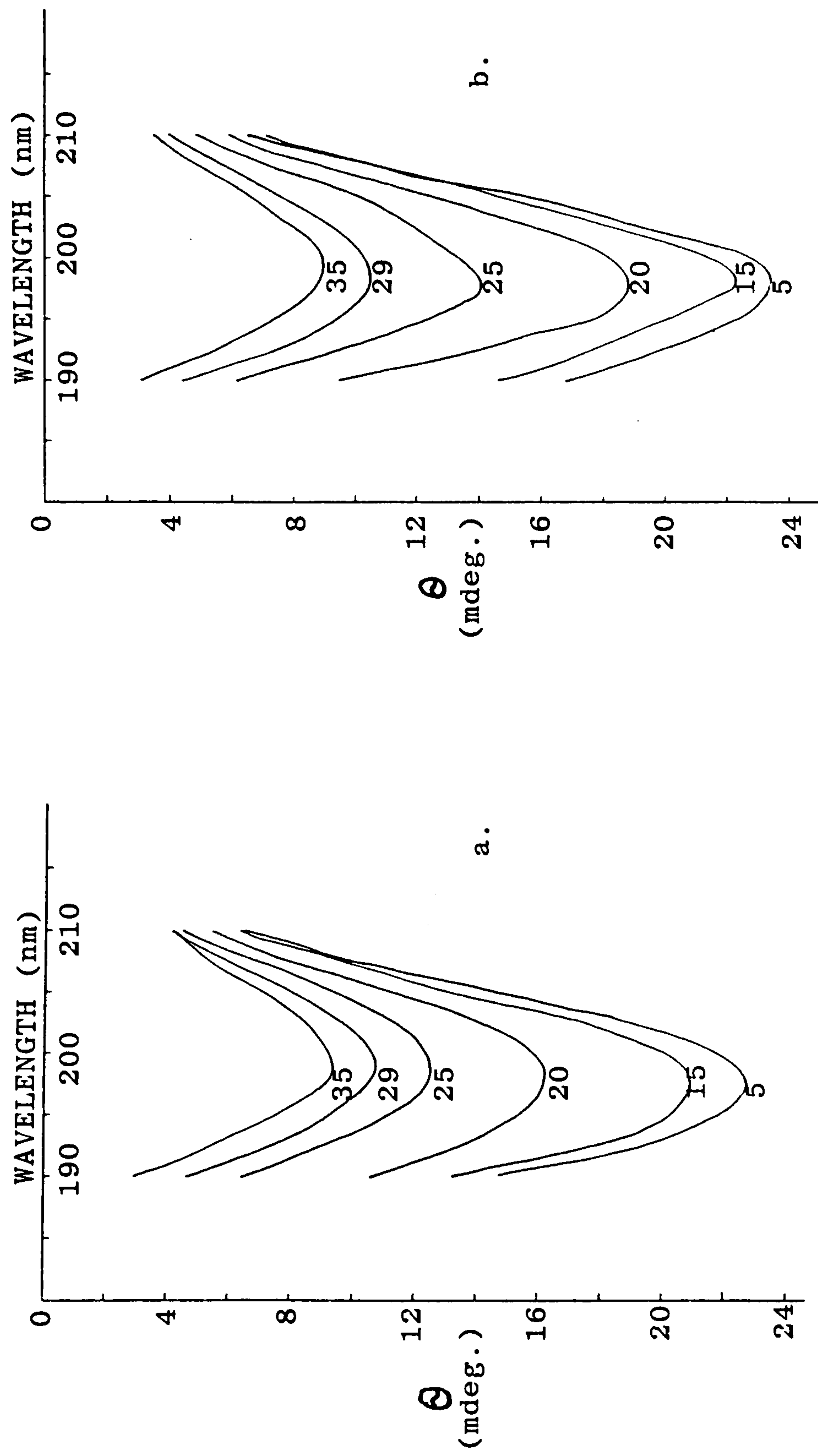


Figure 5.12. Temperature-dependence of the CD spectra of LO-1 gels ( $0.5 \text{ mg ml}^{-1}$ ) recorded in a cell of path-length  $0.1 \text{ mm}$  and over the wavelength range  $\lambda = 190\text{--}210 \text{ nm}$ .

a) Sample quenched directly to  $5^\circ\text{C}$  and held at this temperature for 16 hours prior to melting.  
 b) Sample quenched to  $20^\circ\text{C}$  for 24 hours before being cooled to  $5^\circ\text{C}$  for a further 16 hours and melted. The numbers on the curves are the temperatures ( $^\circ\text{C}$ ) at which the spectra were recorded.

between the two 'difference spectra', indicating that the high-melting and low-melting species have essentially the same ordered structure.

Circular dichroism at lower wavelengths ( $<210$  nm) was also used to monitor the conformational change on heating. The CD spectra of gelatin solutions and gels have a large negative peak at  $\sim 198$  nm which gives rise to the negative values of optical rotation at higher wavelengths. On melting a gelatin sample, the magnitude of this peak (Fig. 5.12 a and b) decreased in a manner corresponding to that of the decrease in peak height at 221 nm. Again, providing no evidence of any specific residue interactions causing the observed higher melting peaks in DSC, optical rotation and rigidity measurements.

#### 5.5.2. Increased length of helices

A second and possibly more likely interpretation of the observed bimodal melting behaviour is that the minimum critical sequence length required for helix formation increases with increasing temperature, so that the helices formed at higher temperature ( $T_0$ ) are longer than those formed on subsequent cooling and therefore melt at higher temperatures (Cantor and Schimmel, 1980). This effect arises from the increased co-operativity in

the formation and melting of longer helices due to the balance between gain in enthalpy and loss of entropy, discussed in Chapter 1 (see also Chapter 7).

#### DSC on samples other than LO-1

In addition to the DSC on sample LO-1, the other seven gelatin samples were also subjected to different cooling regimes, and their melting profiles monitored by DSC. The DSC thermograms for the seven samples are shown in Figs. 5.13 to Fig. 5.19 inclusive (for exact details of the curing conditions see Figure legends) and provide numerous points of interest.

As was the situation with LO-1, the overall enthalpy change for any particular gelatin was approximately constant regardless of the cooling regime used. However, the enthalpies of melting varied considerably for the different samples and are listed in Table 5.3. There is qualitative agreement between the gel strengths of the different samples (Table 4.1) and the enthalpies of melting obtained after 16 hours at 5°C with the increase in  $G'$  paralleling the increase in enthalpy of melting.

All eight gelatin samples show the appearance of higher melting peaks with increased holding time at higher temperatures; but, of greater significance is the fact that, for any particular  $T_0$ , the  $T_m$  of the higher



melting peak is approximately the same for all the samples, whether they be acid processed, lime processed, 1st extract, etc. The size of the higher melting peaks for the samples LO-4, AP-3 and AP-4 (Figs. 5.15, 5.18 and 5.19) are considerably smaller than for the other samples. This seems to support qualitatively the proposal that higher stability is a consequence of longer helices since these samples have a larger proportion of chains of low molecular weight, as measured by GPC, electrophoresis and intrinsic viscosity (Chapter 4), and would therefore be limited in the number of longer helices they might be capable of forming. Also, for these three samples, gels cured at 5°C only have lower melting points than for the other samples, which again could be the result of chainlength distribution.

The sample AP-1 (Fig 5.16) differs somewhat from the other samples in that the melting temperatures are slightly higher, and also there is a larger amount of helix formed at 25°C. This again can be attributed to the molecular weight distribution which, as indicated by electrophoresis (Fig. 4.4), is composed of various discrete chainlengths of relatively high molecular weight. Furthermore, the melting endotherms of AP-1 show discrete waves of melting superimposed on the overall envelope, which is a probable consequence of the rather specific, banded chainlength distribution.

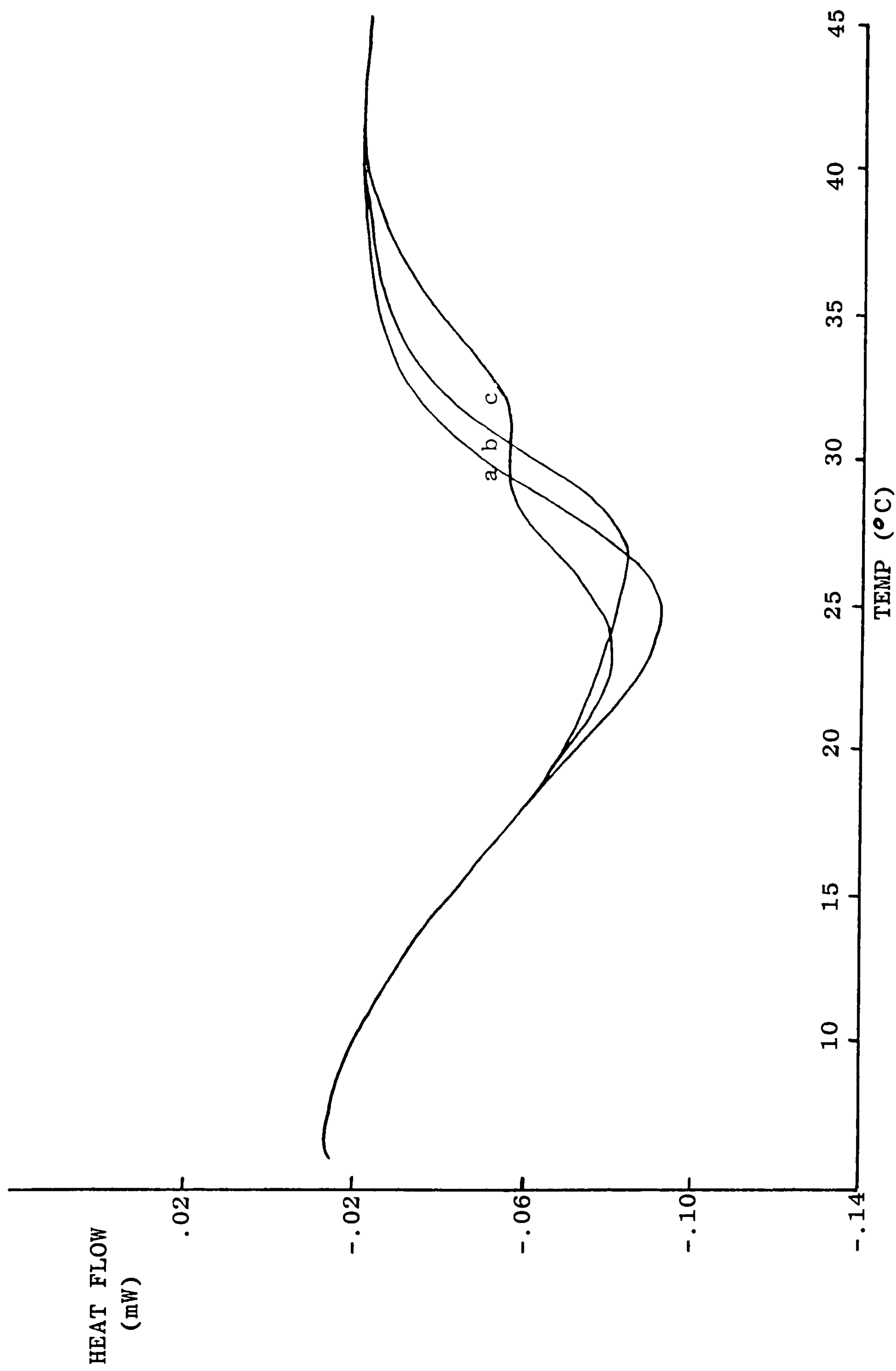


Figure 5.13. DSC melting profiles of LO-2 gels ( $20 \text{ mg ml}^{-1}$ ) pre-matured at various temperatures for varying lengths of time prior to aging at  $5^\circ\text{C}$  for 16 hours.

- a) 16 hours at  $5^\circ\text{C}$  only
- b) 2 hours at  $20^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$
- c) 24 hours at  $25^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$ .

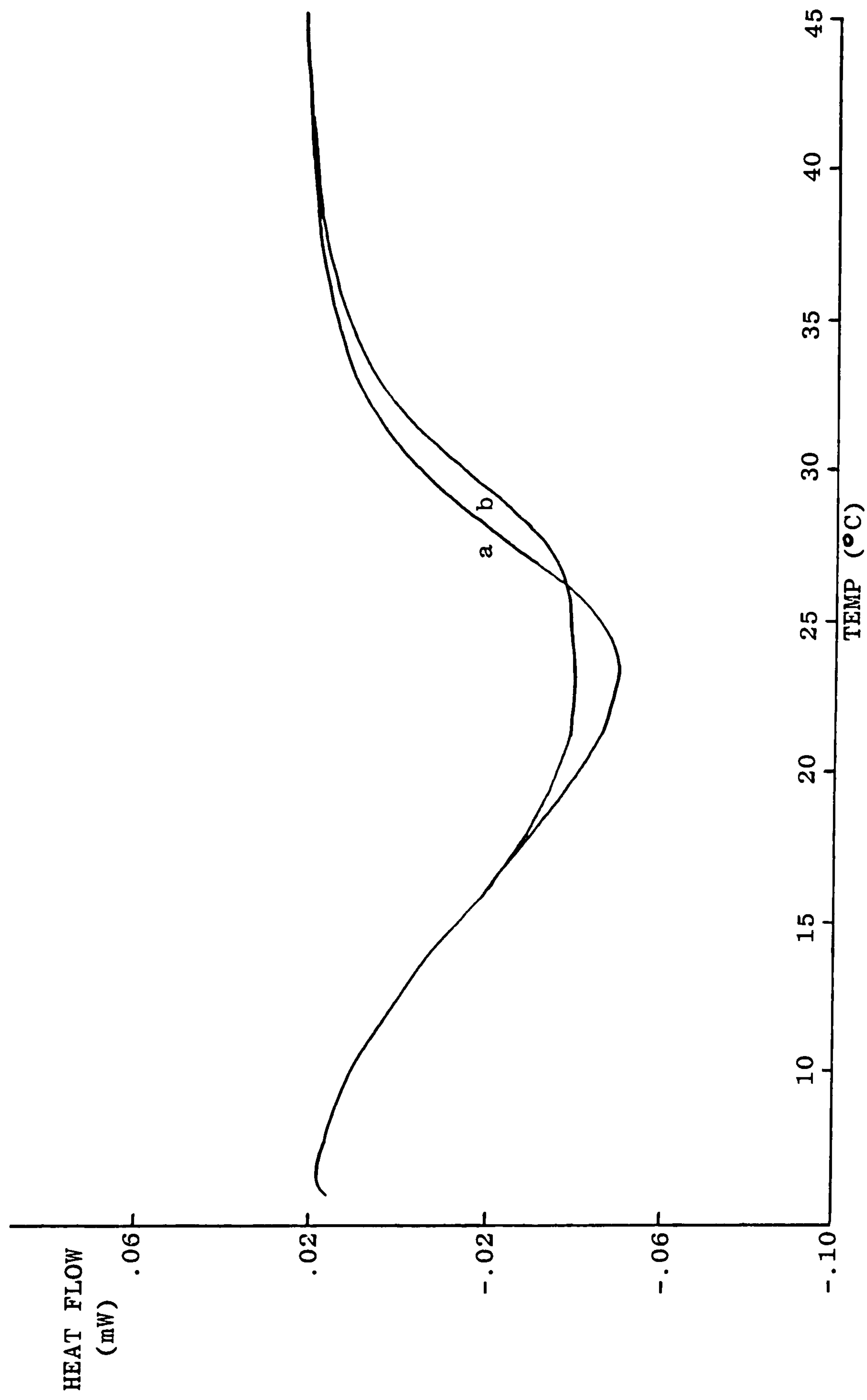


Figure 5.14. DSC melting profiles of LO-3 gels ( $20 \text{ mg ml}^{-1}$ ) pre-matured at  $20^\circ\text{C}$  for 2 hours followed by 16 hours at  $5^\circ\text{C}$  (b) and melted after 16 hours at  $5^\circ\text{C}$  only (a).



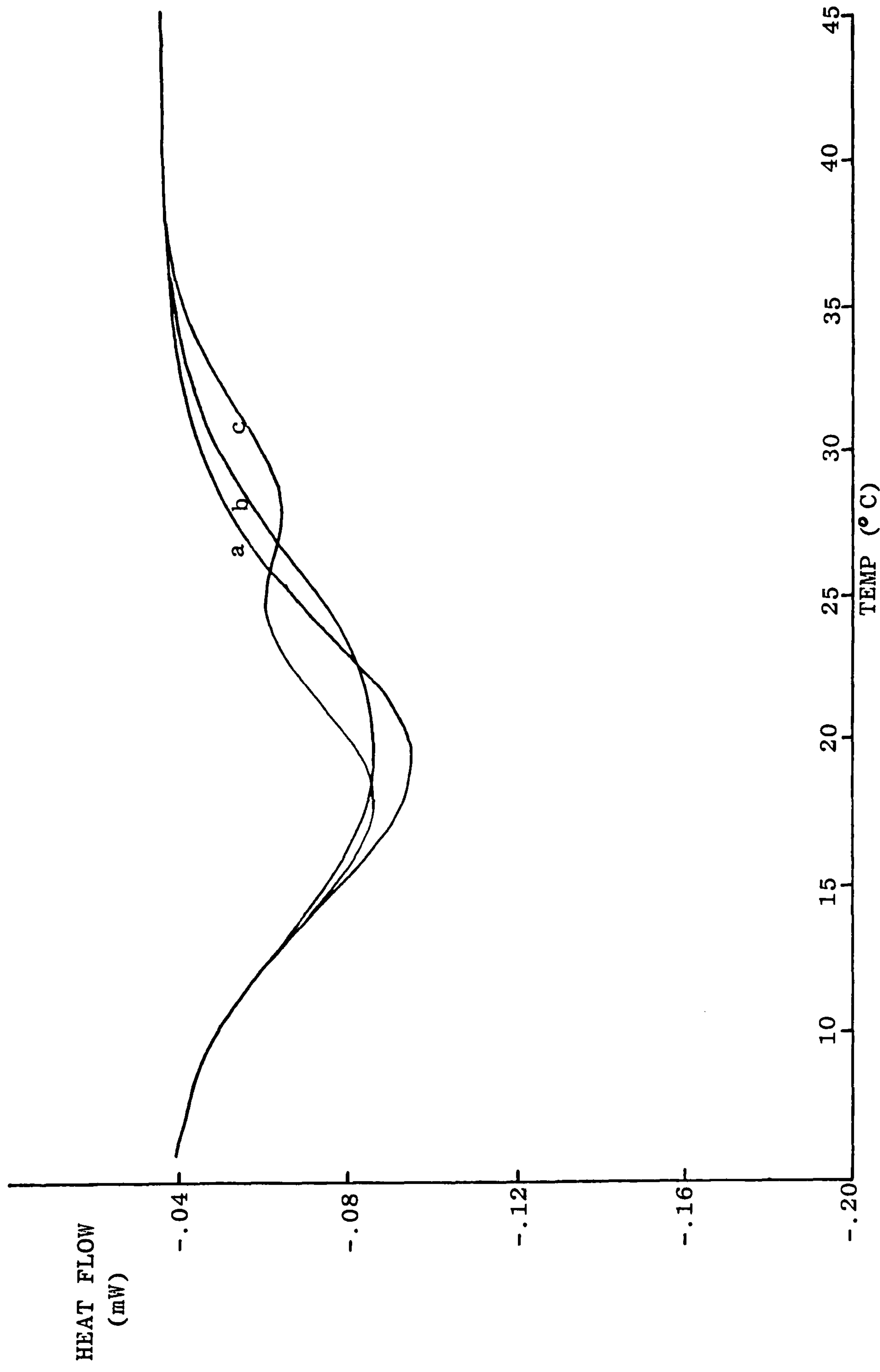


Figure 5.15. DSC melting profiles of LO-4 gels (20 mg ml<sup>-1</sup>) pre-matured at various temperatures for varying lengths of time prior to aging at 5°C for 16 hours  
a) 16 hours at 5°C only  
b) 2.5 hours at 15°C followed by 16 hours at 5°C  
c) 24 hours at 20°C followed by 16 hours at 5°C.

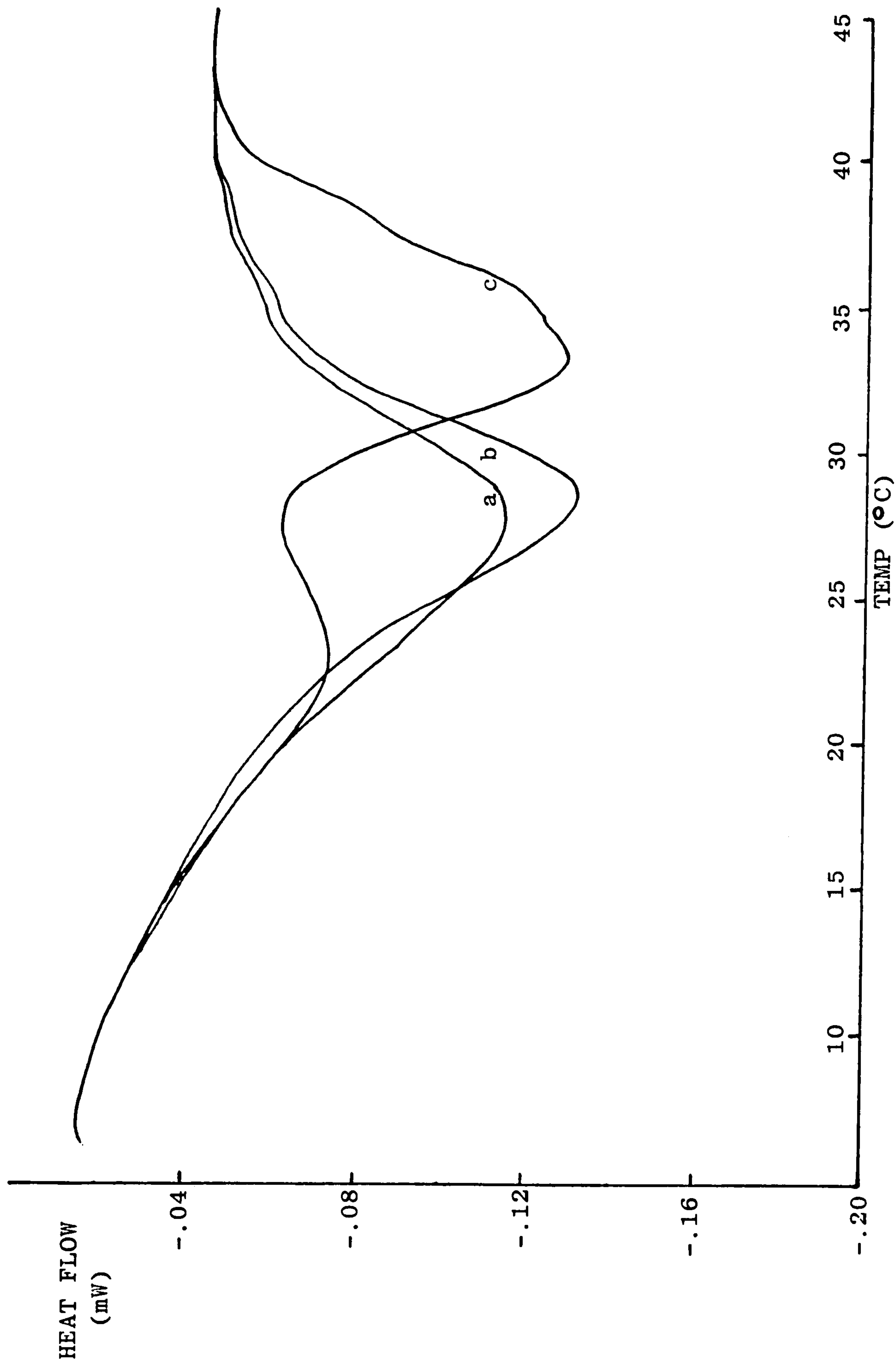


Figure 5.16. DSC melting profiles of AP-1 gels ( $20 \text{ mg ml}^{-1}$ ) pre-matured at various temperatures for varying lengths of time prior to aging at  $5^\circ\text{C}$  for 16 hours  
a) 16 hours at  $5^\circ\text{C}$  only  
b) 1.5 hours at  $15^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$   
c) 72 hours at  $25^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$ .

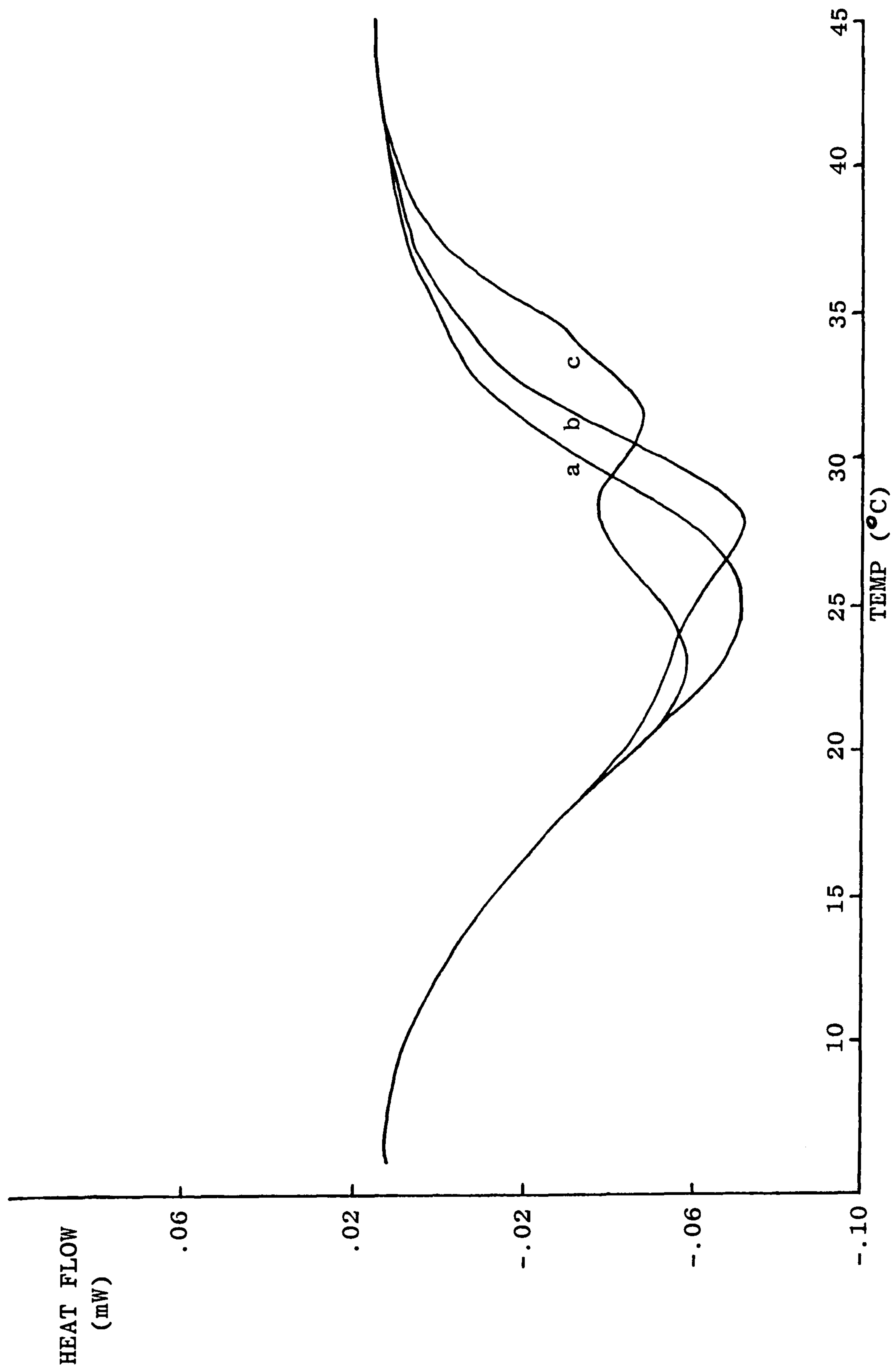


Figure 5.17. DSC melting profiles of AP-2 gels ( $20 \text{ mg ml}^{-1}$ ) pre-matured at various temperatures for varying lengths of time prior to aging at  $5^\circ\text{C}$  for 16 hours  
 a) 16 hours at  $5^\circ\text{C}$  only  
 b) 2 hours at  $20^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$   
 c) 24 hours at  $25^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$ .



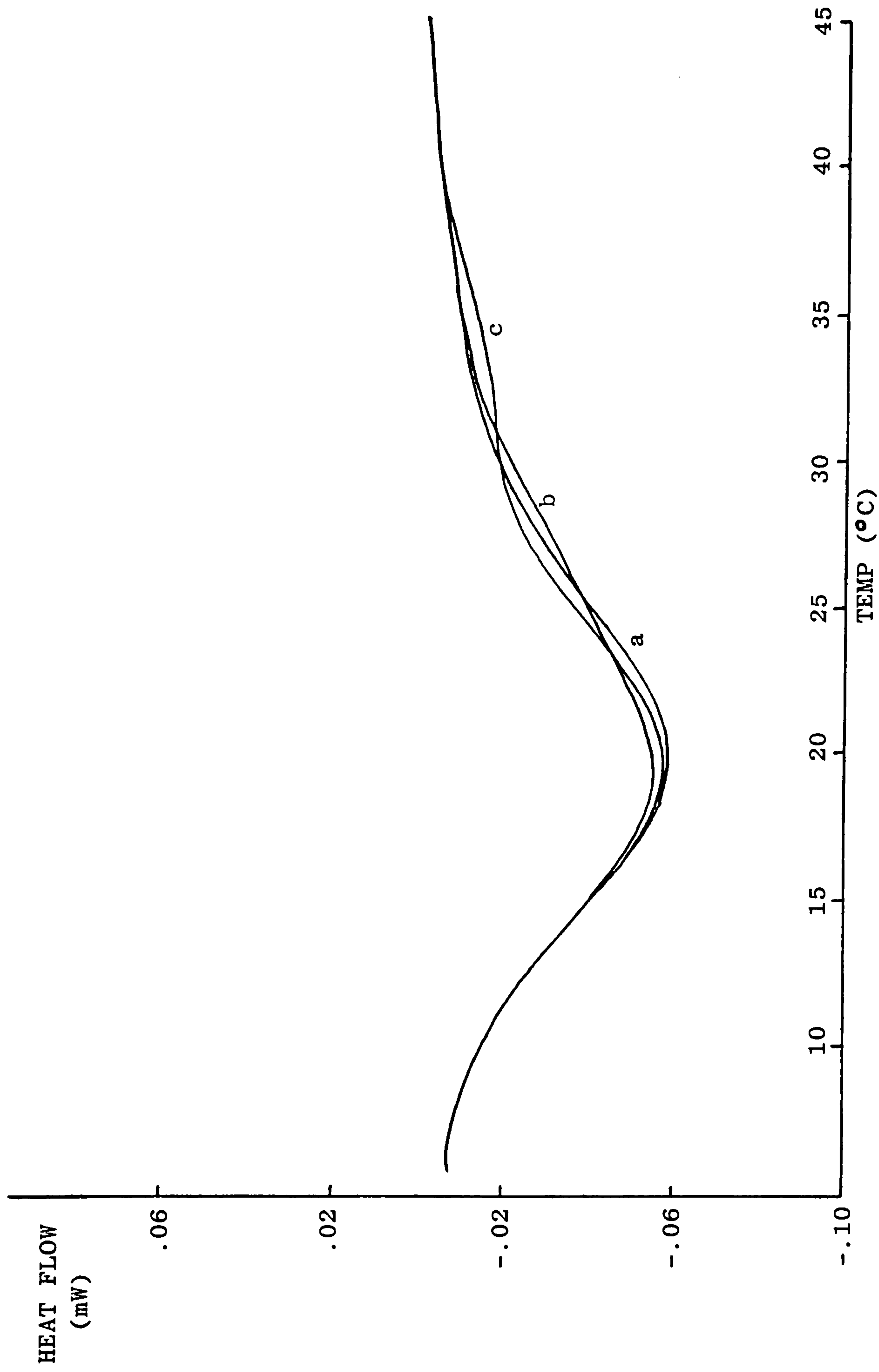


Figure 5.18. DSC melting profiles of AP-3 gels ( $20 \text{ mg ml}^{-1}$ ) pre-matured at various temperatures for varying lengths of time prior to aging at  $5^\circ\text{C}$  for 16 hours  
 a) 16 hours at  $5^\circ\text{C}$  only  
 b) 2 hours at  $20^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$   
 c) 24 hours at  $25^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$ .

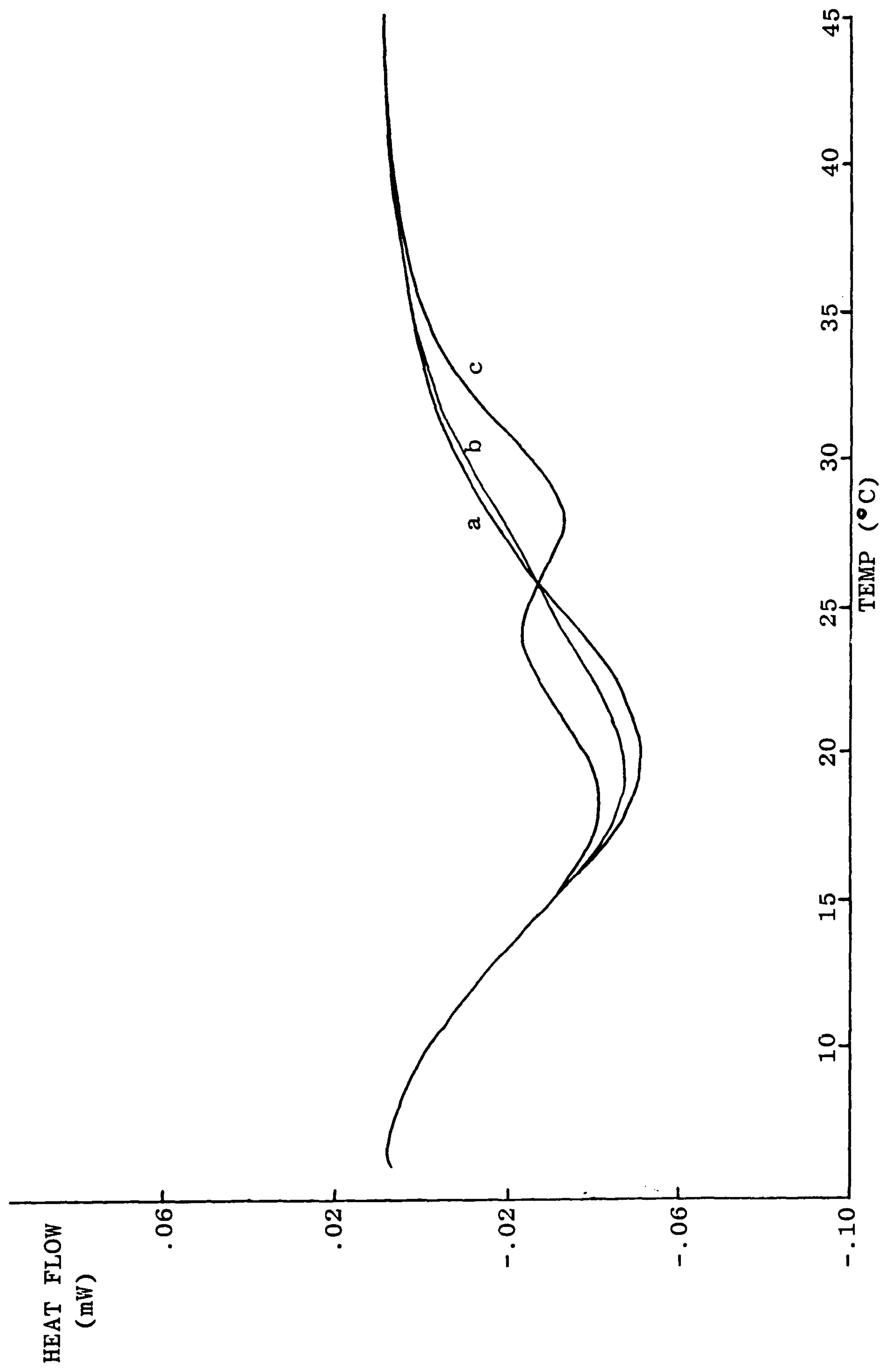


Figure 5.19. DSC melting profiles of AP-4 gels ( $20 \text{ mg ml}^{-1}$ ) pre-matured at various temperatures for varying lengths of time prior to aging at  $5^\circ\text{C}$  for 16 hours  
 a) 16 hours at  $5^\circ\text{C}$  only  
 b) 2 hours at  $20^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$   
 c) 26 hours at  $20^\circ\text{C}$  followed by 16 hours at  $5^\circ\text{C}$ .

Table 5.3. Ethalpies of melting of the eight different samples (concentration 20 mg ml<sup>-1</sup>) after being cured at 5°C for 16 hours.

SAMPLE	ENTHALPY OF MELTING (J/g SOLUTION)	ENTHALPY OF MELTING <sup>a</sup> (kJ/mol)
LO-1	0.652 ±0.02	3.26 ±0.1
LO-2	0.570 "	2.85 "
LO-3	0.588 "	2.94 "
LO-4	0.438 "	2.19 "
AP-1	0.688 "	3.44 "
AP-2	0.711 "	3.56 "
AP-3	0.526 "	2.63 "
AP-4	0.513 "	2.56 "

a Calculated assuming an average amino acid residue molecular weight of 100 for the gelatins.



## 5.6. CONCLUDING REMARKS

From the results presented above it is obvious that curing gels at higher temperatures leads to the formation of populations of triple helices of higher thermal stability, which remain essentially unchanged on dropping to lower temperature. The precise nature of this increased stability has been investigated and there is no evidence to support stabilization through aggregation of helices, or stabilization due to specific amino acid interactions within the helical structure. Examination of different gelatin samples gives qualitative support to the proposal that differences in thermal stability arise from differences in distribution of helix length. However, if helix length is the controlling factor in causing the higher melting behaviour, then the question arises of why there is an associated increase in gel rigidity. As the helix fraction for samples pre-cured at higher temperature and cured only at low temperature remains essentially constant, longer helices would of necessity imply fewer helices for the sample held at the higher temperature, and therefore, at first sight, a weaker network rather than the more rigid structure observed experimentally (Fig. 5.5a).

In Chapter 6 further evidence from other lines of investigation, particularly kinetic studies, presents a unifying interpretation of these apparently conflicting observations.

## CHAPTER 6: KINETICS OF GELATIN GELATION

### 6.1. INTRODUCTION

The mechanism of formation of gelatin gels has been the subject of numerous studies and there are many papers in the literature referring to the kinetics of the process. An early example of one such investigation is that of Smith (1919) who used optical rotation measurements to study the renaturation process, and since then the use of optical rotation in conjunction with other measurement procedures (e.g. viscosity and shear modulus determinations, NMR and light scattering) has formed the basis of much of the work reported.

Ferry (1948b) described the optical rotation of gelatin solutions cooled below 40°C in terms of two phases of optical rotation change, an initial stage lasting several hours, followed by a much slower phase, which continued to progress even after several days. The explanation of such results has proved to be extremely difficult, especially interpretation of the slower phase, during which the laevorotation never appears to reach a constant value. The increase in laevorotation is attributed to renaturation of the collagen triple helical structure, but the slowness of this process, compared to the thermal denaturation of intact collagen monomers, clearly argues against a simple, two state,



all coil or all helix mechanism for the transition. Indeed, on renaturation, the values of optical rotation never reach those of the intact collagen.

In one mechanistic study (Flory and Weaver, 1960) gelatin solutions, below their gelling concentration, were rapidly quenched to temperatures in the range 5° to 23°C. It was shown that the amount of renaturation was extremely dependent on temperature, with far less recovery taking place at higher temperatures than at lower temperatures. From analysis of the results, it was concluded that the regeneration of the triple helical structure was, surprisingly, a first order process, with the reaction rate showing a high negative temperature dependence. In order to explain the first order concentration dependence, Flory and Weaver proposed a model for helix formation in which the rate determining step involved the transient formation of helical segments in the proline-rich parts of gelatin chains, with rapid conversion of these structures to the triple helix through intermolecular reaction. The effect of temperature on the reaction rate (R) was also discussed and described by the equation

$$\ln R = -A/T\Delta T \quad (\text{Equation 6.1})$$

where  $\Delta T$  is the extent of undercooling below the melting temperature,  $T_m$ , of the parent collagen.



This type of relationship is characteristic of polymer crystallization with apparent negative energy and entropy of activation (Rose, 1986).

At approximately the same time as the Flory-Weaver work, Harrington and von Hippel (1961) confirmed the concentration-dependence of specific rotation data at low gelatin concentrations. They, however, proposed a kinetic mechanism involving a very rapid initial folding of the proline-rich segments into an ordered conformation, followed by a slower and more complete ordering of individual chains and finally slow association of the chains into triple helices. This mechanism is markedly different to the Flory-Weaver mechanism, in that the individual ordered chains are ascribed an intrinsic stability, explained in terms of water molecules becoming involved in stabilisation through hydrogen bonding.

These two proposed mechanisms for helix formation were debated for a number of years but in the light of further experimental work, particularly on purified gelatins, have gradually become superceded by more detailed interpretations of results. The explanation of the unimolecular kinetics by the Flory-Weaver intermediate became unnecessary, as antiparallel reverse helix formation within single chains was envisaged at low concentrations, with intermolecular helix formation becoming more probable at higher concentrations.

Detailed proposals have been advanced (Harrington and Rao, 1970; Harrington and Karr, 1970; Yuan and Veis, 1973) to explain the later stages of helix renaturation in terms of nucleation, growth and annealing processes with markedly different rate constants and temperature dependences. At low quenching temperatures, it is proposed that nucleation is fast, growth is slower and annealing processes occur very slowly, whereas at higher temperatures the growth process dominates, thus altering the nature of the helices formed. Another kinetic limitation to helix formation described more recently (Bruckner *et al.*, 1981) involves the *cis-to-trans* isomerisation of peptide bonds, particularly at imino acid residues, which have a greater likelihood than other amino acids of existing in the *cis* form, due to their cyclic secondary amine structure (see Chapter 1).

Various mechanisms have been proposed to explain the relationship between helix renaturation and gel formation. Harrington and Rao (1970) suggested that, in semi-dilute solutions, the renaturation of the helices involved the formation of triple helical segments composed of strands of different gelatin chains; thus development of a 3-dimensional mesh-like structure with chains involved in more than one helical segment could occur. An alternative model (Eagland *et al.*, 1974) preferred intramolecular helices, even at high concentrations, and proposed gel formation through



association of ordered species via entanglements, van der Waals interactions and hydrogen bonds. Finally, Godard *et al.*, (1978) viewed gelation as a crystallisation phenomenon, the junction zones in the gel network being fibre-like assemblies formed by lateral association of triple helical structures. Although some electron microscopy evidence appears to support the latter theory (Godard *et al.*, 1978; Tomka *et al.*, 1975), the generally accepted model at present is the inter-chain triple helix scheme of Harrington and Rao.

Despite the large number of investigations that have been carried out on gelatin gelation, there is still no analytical theory for the renaturation process that will predict all the behaviour observed experimentally. Recently, Djabourov *et al.*, (1983, 1985) have fitted optical rotation data for quenched gelatin solutions using a combination of Avrami crystallisation kinetics and a logarithmic time-dependence. The processes involved have been identified by Djabourov and co-workers as a nucleated polymer-crystallisation step coupled with secondary crystallisation at longer times. Although this treatment fits the experimental data quite well, it gives no indication of what is actually occurring at a molecular level.



The remainder of this chapter deals with a detailed re-examination of the kinetics of the initial stages of renaturation of gelatin, using optical rotation as a measure of helix fraction. Although the kinetics of the gradual increase in helix fraction at long times after quenching are particularly complex, in the very early stages of renaturation, before any substantial amount of helix has formed, helix content increases linearly with time; thus, the initial slope of reaction progress curves can be obtained, and provide a direct measure of the rate of helix formation under conditions where competing reactions (e.g. denaturation of helices once formed) have negligible effect.

## 6.2. SPECIFIC EXPERIMENTAL DETAILS

The reaction progress curves for conformational ordering of a gelatin solution (LO-1; 20 mg ml<sup>-1</sup>), on quenching to various temperatures, are illustrated in Fig. 6.1 in the form of a plot of helix fraction versus log time. For samples cured at high temperatures (20, 25°C) it can be seen that the rate of helix formation was extremely slow, making these temperatures experimentally impractical for initial rate measurements, especially at the lower concentrations studied. On the other hand, however, with low quench temperatures (5, 10°C), the initial stages of helix regeneration were over so quickly that it was impossible to obtain reliable

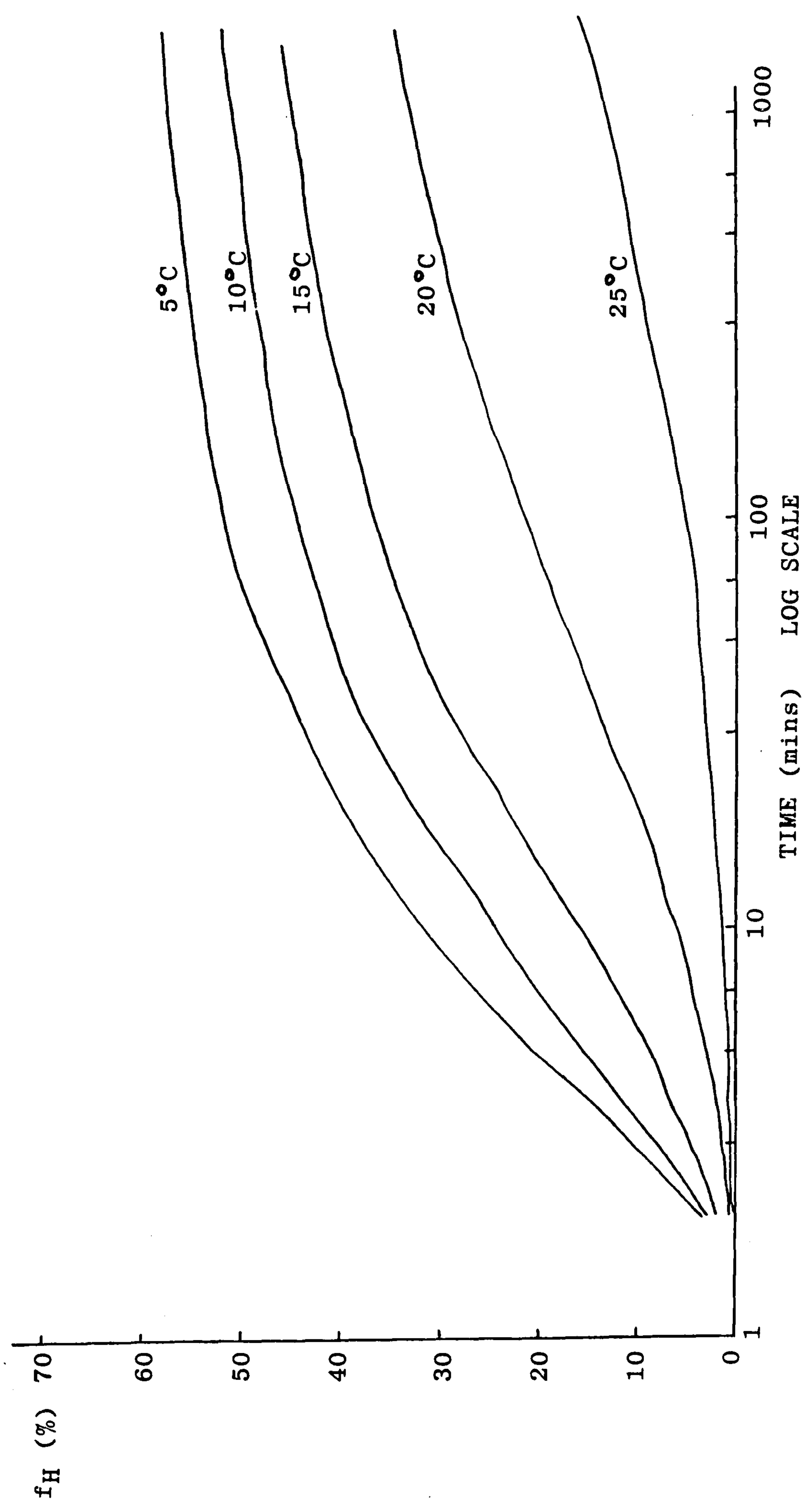


Figure 6.1 Regeneration of helix fraction (monitored by optical rotation) as a function of time (illustrated on a log scale) on quenching an LO-1 solution (20 mg ml<sup>-1</sup>) to temperatures of 5, 10, 15, 20 and 25°C.

results, particularly as a significant amount of ordered structure had formed during the time required to cool to the final quench temperature. For these reasons an experimental quench temperature of 15°C was chosen as the best compromise for the whole concentration range studied throughout this kinetic investigation.

In order to minimise the length of time required to reach 15°C, the sample was injected at 45°C into a polarimeter cell pre-cooled with water at 5°C; the temperature of the sample was constantly monitored with a thermocouple, and on reaching 15°C the water bath circulating water at 5°C was replaced by a second water bath circulating water at 15°C, using a system of taps (see Fig. 6.2). Using this technique it was possible to attain a sample temperature of 15°C within 30 seconds, during which time no significant conformational ordering had occurred.

In order to maximise the concentration range, it was necessary to take optical rotation measurements at different wavelengths and in different pathlength cells. At extremely low concentrations ( $<0.5 \text{ mg ml}^{-1}$ ), the wavelength used was 255 nm in conjunction with a 10 cm supracil cell; this was to give the highest possible optical rotation readings. Above  $0.5 \text{ mg ml}^{-1}$ , however, transmission at 255 nm became very poor and the wavelength of measurement had to be changed to 365 nm. Finally, at very high concentrations ( $>10 \text{ mg ml}^{-1}$ ),



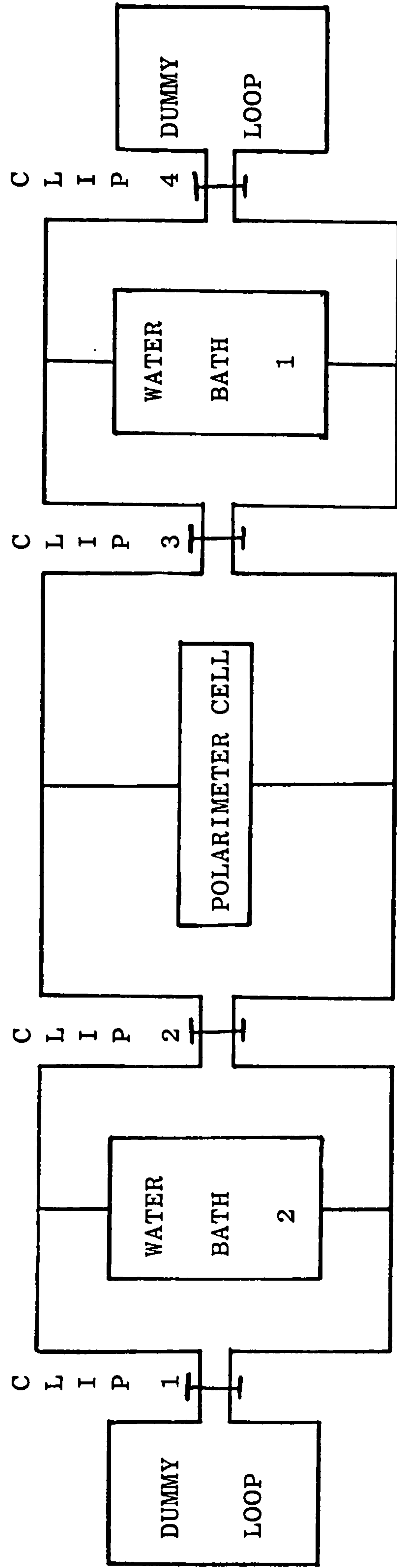


Figure 6.2 Schematic diagram of the system used to quench the gelatin samples to 15°C at the fastest possible rate. The polarimeter cell was maintained at low temperature via water bath 1 (clips 2 and 4 closed; clips 1 and 3 open). The sample was then injected into the cold cell and when the sample temperature reached 15°C, water bath 1 was replaced by water bath 2 (circulating water at 15°C) by opening clip 4, closing clip 3, opening clip 2 and closing clip 1.

there was insufficient transmission through the 10 cm cell and the pathlength was therefore reduced to 1 cm.

### 6.3. CONVERSION OF OPTICAL ROTATION DATA TO REACTION PROGRESS CURVES

Changes in helix fraction during the renaturation of gelatin after quench cooling were calculated from optical rotation data using values of specific rotation,  $[\alpha]$ , for the 'all-helix' and 'all-coil' forms at the same temperature and wavelength. Values of  $[\alpha]$  for the coil form,  $[\alpha]_c$ , were obtained by direct measurement at temperatures above the onset of helix formation, and were found to be independent of concentration and to increase linearly with decreasing temperature, as illustrated in Fig. 6.3. Taking the specific rotation at 45°C,  $[\alpha]_{45}$ , as a reference value, values of  $[\alpha]$  for the 'all-coil' form at lower temperatures,  $[\alpha]_{cr}$ , could then be obtained from a linear equation of the form:

$$[\alpha]_{cr}/[\alpha]_{45} = 1 + G(45 - T) \quad (\text{Equation 6.2})$$

where  $T$  is temperature (°C) and  $-G$  is the gradient of  $[\alpha]/[\alpha]_{45}$  vs.  $T$  at  $T > 40^\circ\text{C}$ . Values of  $[\alpha]_{45}$ ,  $G$ , and  $[\alpha]_{cr}$  used in calculations of helix fraction from optical rotation are listed in Table 6.1.

The specific rotation of native collagen is reported (Djabourov and Papon, 1983) to be independent of temperature below the onset of helix melting ( $T < 30^{\circ}\text{C}$ ) and to have a value of  $800^{*} \pm 10$  at 436 nm. The corresponding values of specific rotation for the all-helix form,  $[\alpha]_{\text{H}}$ , at the shorter wavelengths used in the present work (365 and 255 nm) were derived from the reported experimental value at 436 nm by the following procedure. The specific rotation of partially renatured solutions (at various times after quenching to  $5^{\circ}\text{C}$ ) was measured at all three wavelengths (436, 365 and 255 nm). Since the 'all-helix' and 'all-coil' values at  $5^{\circ}\text{C}$  and 436 nm are both known ( $[\alpha]_{\text{H}} = 800^{*}$ , from the literature value for native collagen, and  $[\alpha]_{\text{C}} = 275^{*}$ , from Table 6.1), the helix fraction,  $f_{\text{H}}$ , could be derived from the observed specific rotation at this wavelength, by the relationship:

$$[\alpha] = f_{\text{H}}[\alpha]_{\text{H}} + (1-f_{\text{H}})[\alpha]_{\text{C}} \quad (\text{Equation 6.3})$$

$$\text{i.e. } f_{\text{H}} = ([\alpha] - [\alpha]_{\text{C}})/([\alpha]_{\text{H}} - [\alpha]_{\text{C}}) \quad (\text{Equation 6.4})$$

Using this value, the specific rotation for the 'all-helix' form at lower wavelengths (365 and 255 nm) could then be calculated from the 'all-coil' value (Table 6.1) and the net observed value,  $[\alpha]$ , by:

$$[\alpha]_{\text{H}} = [\alpha]_{\text{C}} + ([\alpha] - [\alpha]_{\text{C}})/f_{\text{H}} \quad (\text{Equation 6.5})$$

\* Values of  $[\alpha]$  given in units of mdeg.  $\text{g}^{-1} \text{ dm}^2$ .



TABLE 6.1.

TEMPERATURE-DEPENDENCE OF SPECIFIC ROTATION  
FOR THE 'ALL-COIL' FORM AT VARIOUS WAVELENGTHS ( $\lambda$ )

$\lambda$ (nm)	$[\alpha]_{45}$	G	$[\alpha]_{c5}$	$[\alpha]_{c15}$	$[\alpha]_H$
436	254	.00205	275	270	800
365	418	.00217	454	445	1268*
255	1404	.00276	1559	1520	3798*

\* Calculated in Table 6.2.

TABLE 6.2

DERIVATION OF SPECIFIC ROTATION  $[\alpha]_H$  FOR THE 'ALL-HELIX' FORM  
AT WAVELENGTHS USED IN STUDIES OF GELATIN RENATURATION (365 AND 255 NM)

QUENCH TIME (min)	$[\alpha]_{436}$	$f_H$	365 nm			255 nm		
			$[\alpha]$	$[\alpha]_{cs}$	$[\alpha]_H$	$[\alpha]$	$[\alpha]_{cs}$	$[\alpha]_H$
20	396	0.230	640	454	1262	2075	1559	3802
60	444	0.322	716	454	1267	2278	1559	3792
240	486	0.402	784	454	1275	2460	1559	3800
				mean =	<u>1268</u>		mean =	<u>3798</u>

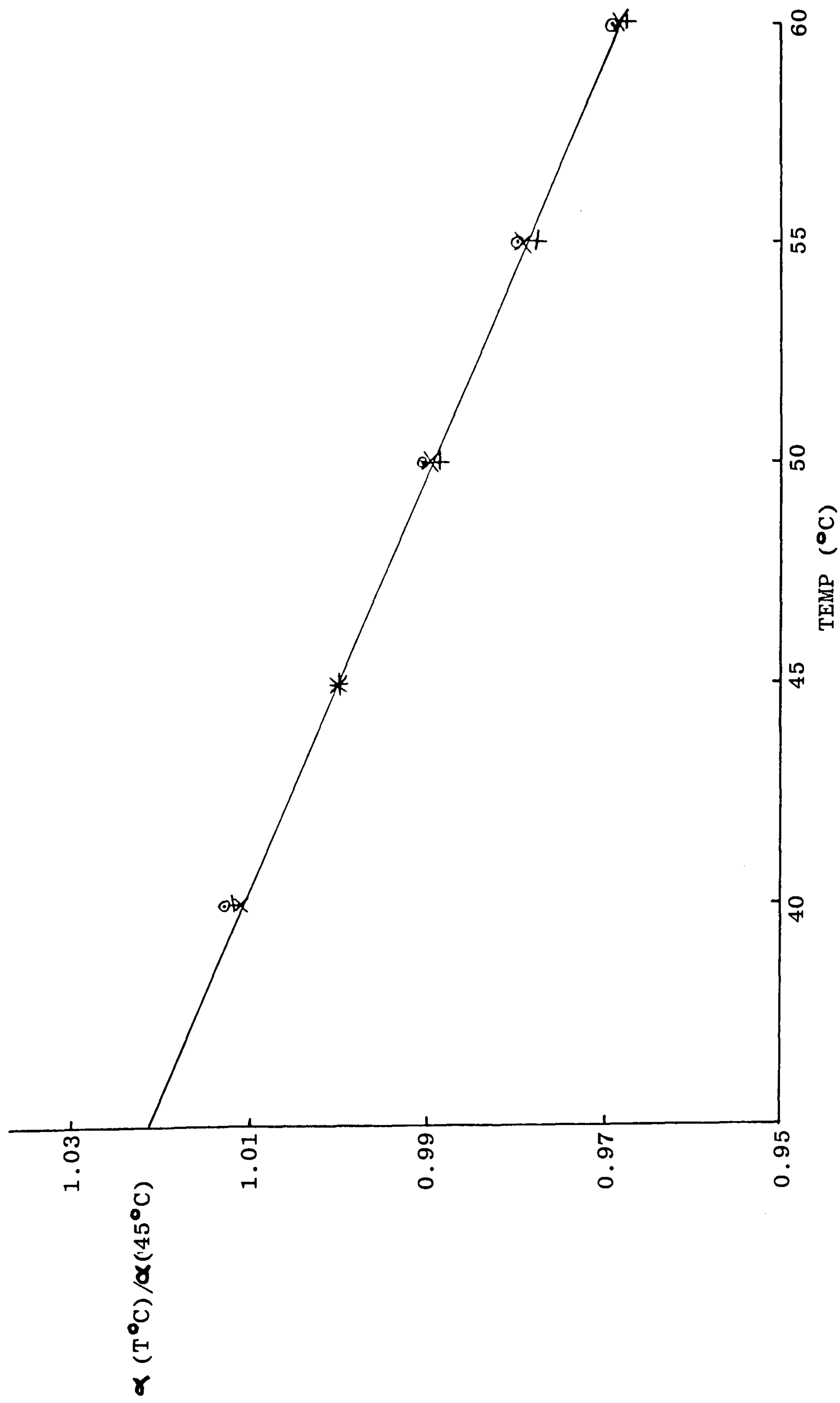


Figure 6.3 The linear plot of optical rotation ( $\lambda = 365 \text{ nm}$ ) relative to the optical rotation at  $45^\circ\text{C}$  as a function of temperature for random coil gelatin:

- ( $\odot$ ) gelatin concentration of  $2 \text{ mg ml}^{-1}$  utilizing a  $10 \text{ cm}$  pathlength cell.
- ( $\times$ ) gelatin concentration of  $10 \text{ mg ml}^{-1}$  utilizing a  $10 \text{ cm}$  pathlength cell.
- ( $+$ ) gelatin concentration of  $50 \text{ mg ml}^{-1}$  utilizing a  $1 \text{ cm}$  pathlength cell.



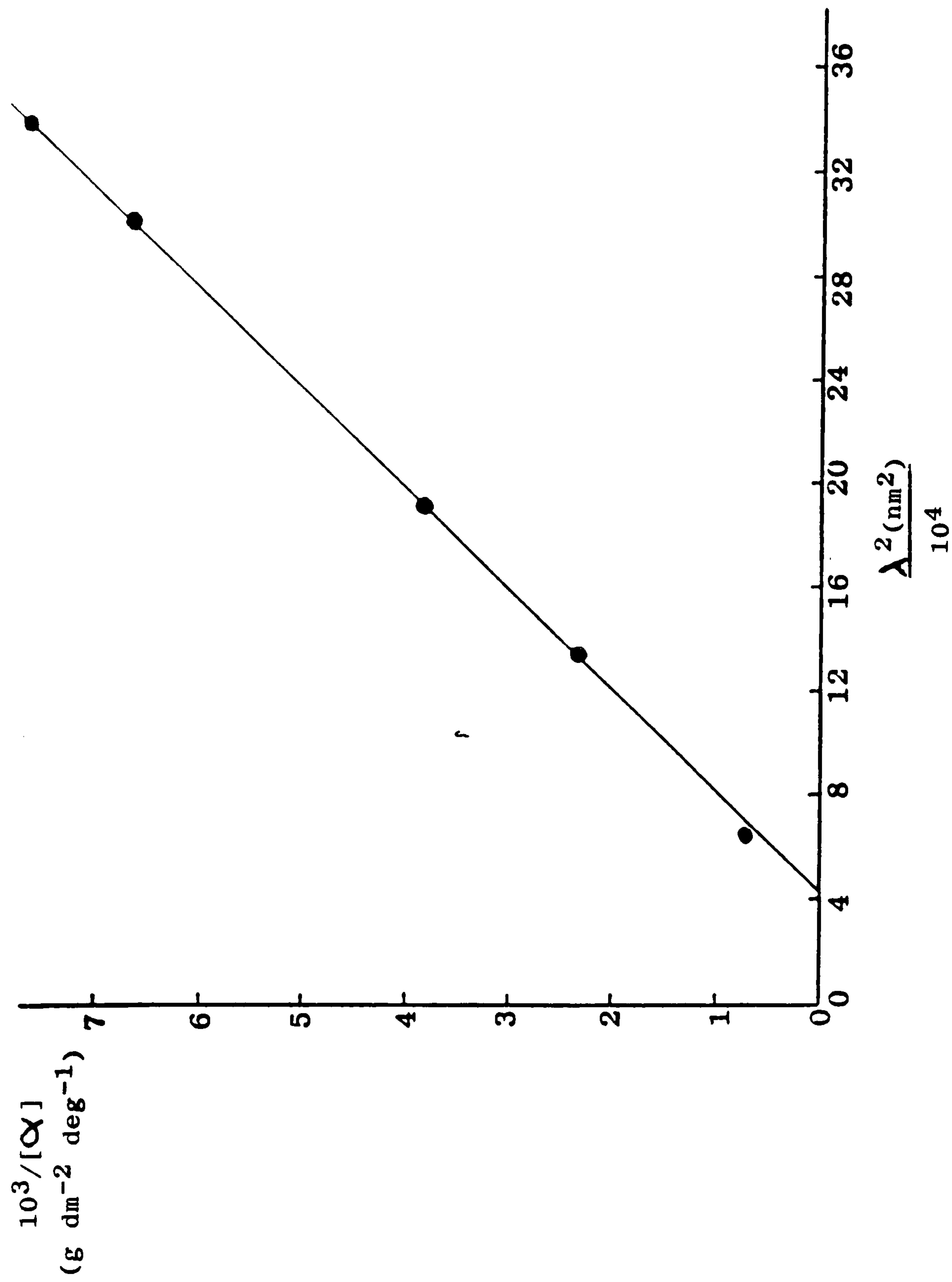


Figure 6.4a. Drude plot showing the wavelength ( $\lambda$ ) dependence of specific rotation,  $[\alpha]$ , for gelatin sample LO-1 in the 'all coil' form at 45°C.

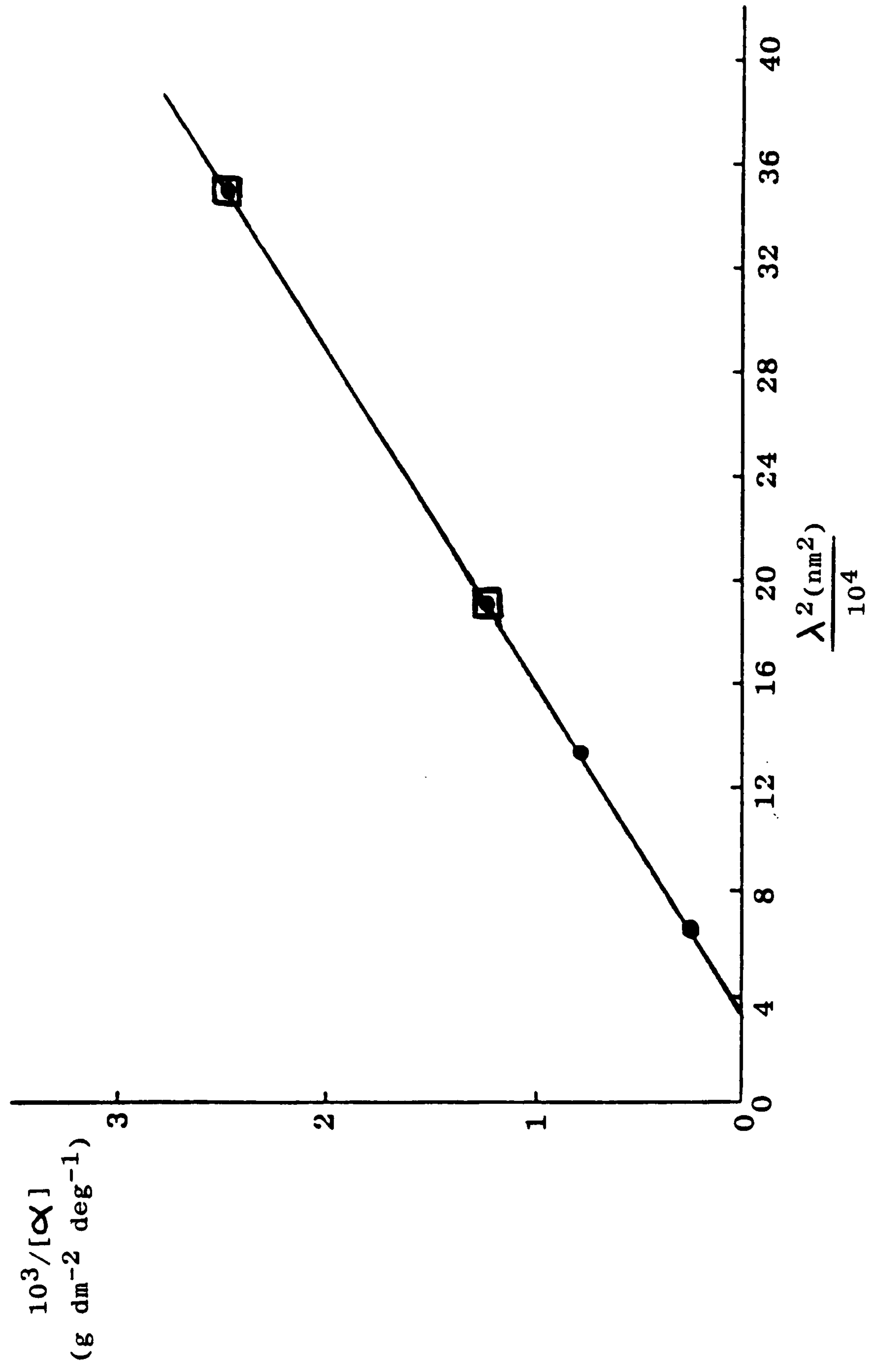


Figure 6.4b. Drude plot for the 'all helix' form of gelatin sample LO-1 (Table 6.1) - circles, and for native collagen (Djabourov and Papon, 1983) - squares.

Values of  $[\alpha]_H$  derived from solutions of different helix fraction (Table 6.2) were in good agreement; the mean of these values for the 'all-helix' form are included in Table 6.1, along with the corresponding values for the 'all-coil' form. Both give linear Drude plots (see Chapter 3) for the wavelength-dependence of optical activity (Fig. 6.4), lending further confidence in the use of the parameters from Table 6.1 to calculate  $f_H$  from optical rotation (Equation 6.4) in studies of the 'initial slope' kinetics for gelatin renaturation.

The renaturation curves, in terms of helix fraction, for each concentration studied are shown in Figs. 6.5a, b, and c. From these plots the initial reaction rates ( $df_H/dt$ ) were obtained by fitting the best line to the linear portion of the reaction curve, which generally began to deviate from linearity at a helix fraction of approximately 6%.

#### 6.4. CONCENTRATION-DEPENDENCE OF ORDER OF REACTION

The initial slopes from the reaction curves discussed in the last section are listed in Table 6.3 and represented graphically as a function of concentration in Fig. 6.6. At concentrations below approximately  $0.5 \text{ mg ml}^{-1}$ , the initial slope ( $S = df_H/dt$ ) remains constant; i.e. at these low concentrations the reaction displays first-order kinetics, consistent with an intra-molecular



process as the rate limiting step. At higher concentrations, however, the value of  $S$  increases substantially (by approximately an order of magnitude over the concentration range studied) indicating the onset of a second mechanism of conformational ordering.

The kinetics of this second process have been examined by subtracting the constant value of  $S$  at low concentrations ( $S_1$ ) from the experimental value of  $S$  at higher concentrations to obtain the contribution ( $S_2 = S - S_1$ ) from the second process. As shown in Fig. 6.6 the slope of  $\log S_2$  versus  $\log c$  is very close to unity (1.04) and thus the additional process at higher concentration appears to be second order, consistent with the involvement of two chains in the rate-limiting step to triple helix formation. The point indicated in Fig. 6.6 where  $S_2 = S_1$  gives the concentration at which the two processes make an equal contribution to the overall reaction rate.

In summary, therefore, the kinetic analysis appears to suggest two mechanisms for conformational ordering. At low concentrations, the process appears to be almost entirely intramolecular while at higher concentrations, a second, bimolecular process, superimposed on the intramolecular process, is evident.

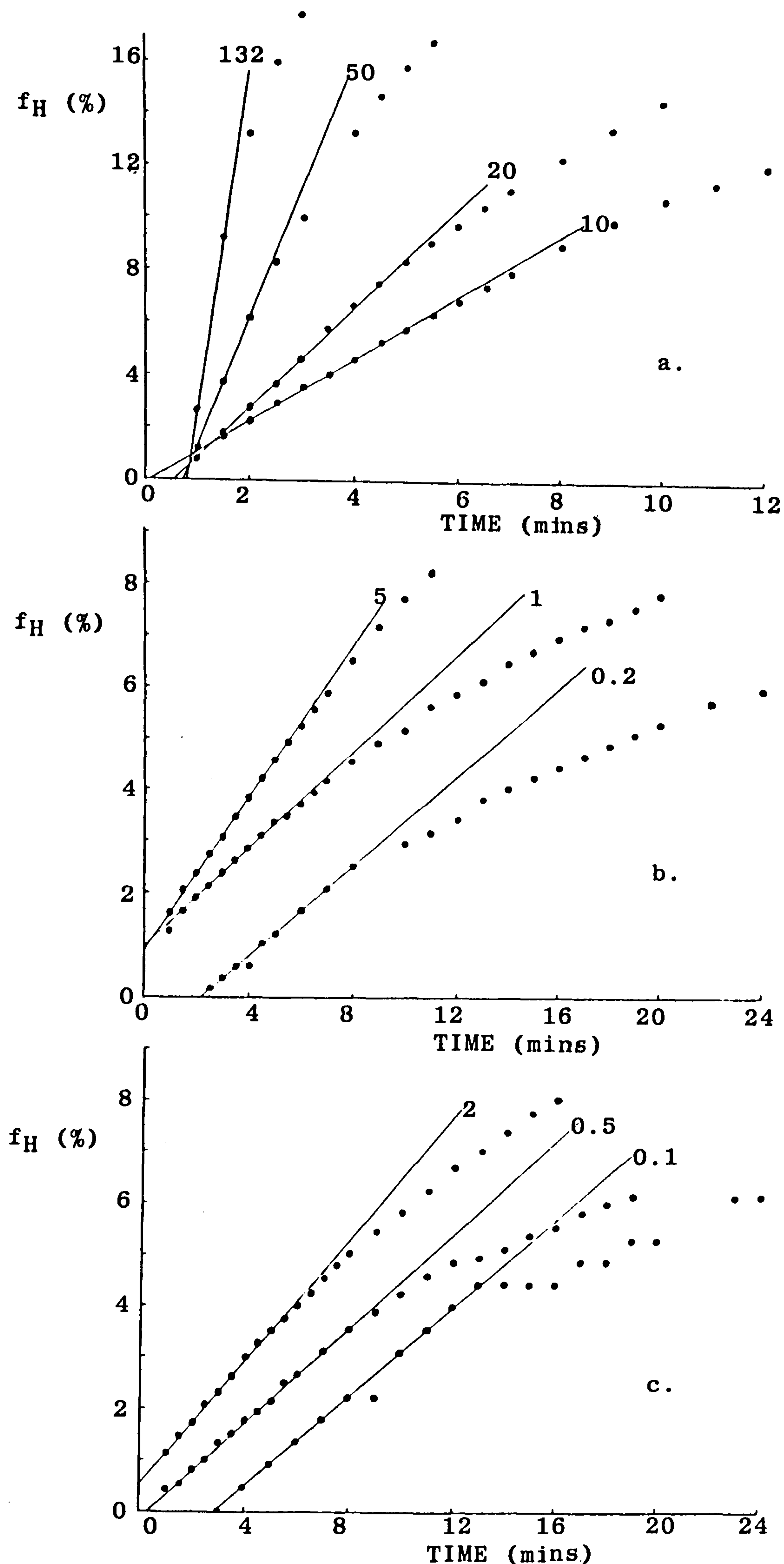


Figure 6.5 Initial rate of helix regeneration (determined from optical rotation) for varying concentrations of LO-1 plotted in terms of helix fraction versus time after quenching the sample to  $15^\circ\text{C}$ :  
 a) LO-1 concentrations of 132, 50, 20 and 10  $\text{mg ml}^{-1}$   
 b) LO-1 concentrations of 5, 1 and 0.2  $\text{mg ml}^{-1}$   
 c) LO-1 concentrations of 2, 0.5 and 0.1  $\text{mg ml}^{-1}$

Also shown are the initial slopes used in the analysis of the 'reaction order' (see Table 6.3).

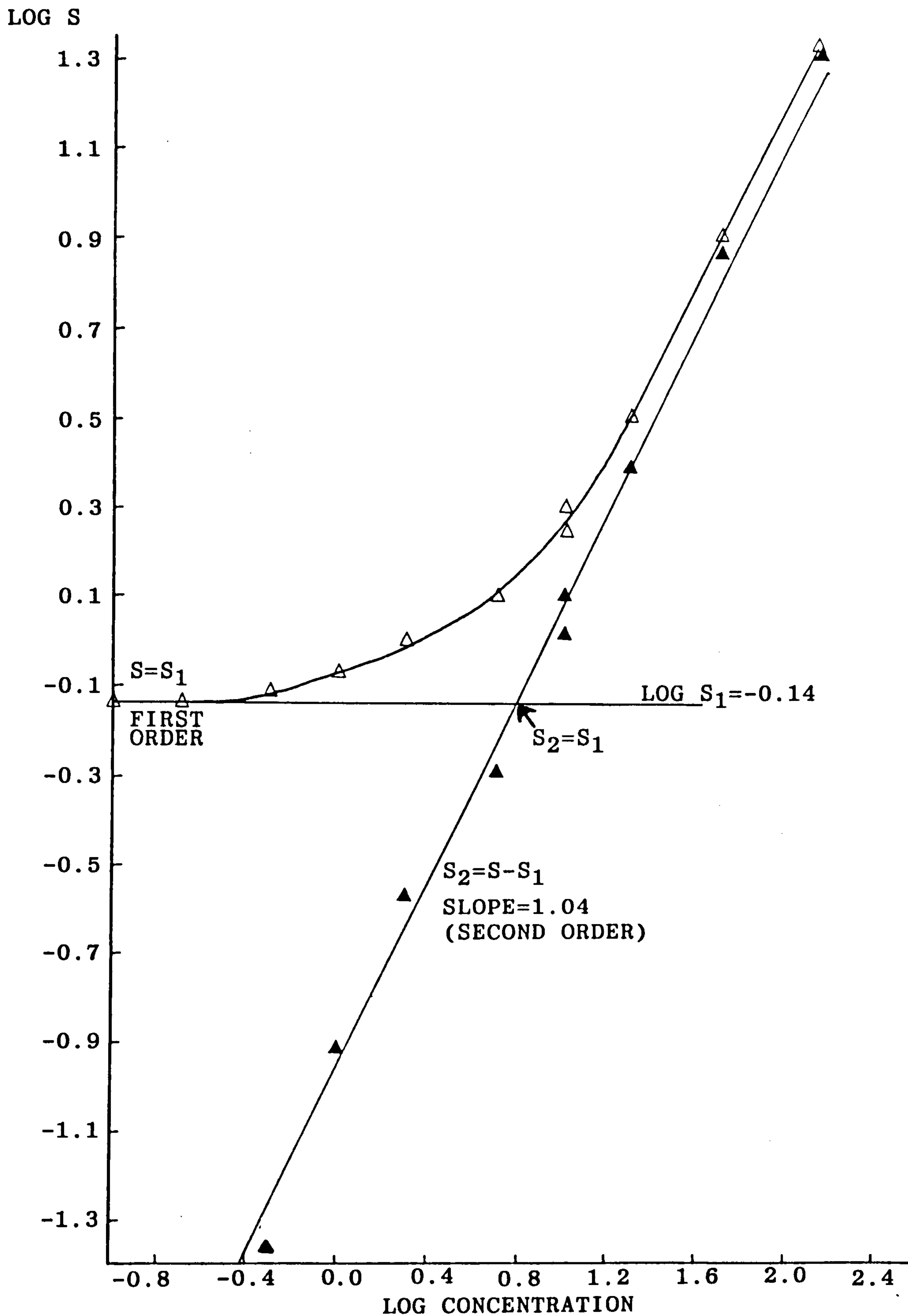


Figure 6.6 Concentration-dependence of 'initial slope' kinetics for renaturation of gelatin at  $15^\circ\text{C}$ . The observed initial slope ( $S = df_H/dt$ ) is plotted as open symbols, with filled symbols representing the contribution of the additional process at higher concentrations in terms of  $S_2 = S - S_1$  where  $S_1$  is the constant value of  $S$  at low concentration.



TABLE 6.3

THE INITIAL SLOPES OF THE REACTION CURVES OBTAINED FROM FIG. 6.5  
ALONG WITH THE CONCENTRATIONS AND VALUES OF  $S_2=S-S_1$ , using a value  
of  $S_1=0.72$  (i.e.  $\log S_1=-0.14$ )

CONC. (C) (mg ml <sup>-1</sup> )	SLOPE (S) (10 <sup>4</sup> S <sup>-1</sup> )	LOG C	LOG S	$S_2=S-S_1$ (10 <sup>4</sup> S <sup>-1</sup> )	LOG S <sup>2</sup>
0.10	0.72	-1.00	-0.14	-	-
0.20	0.72	-0.70	-0.14	-	-
0.50	0.77	-0.30	-0.11	0.04	-1.37
1.00	0.84	0.00	-0.07	0.12	-0.92
2.00	0.99	0.30	0.00	0.27	-0.57
5.00	1.24	0.70	0.09	0.51	-0.29
10.00	1.75	1.00	0.24	1.03	0.00
10.00	1.99	1.00	0.30	1.27	0.10
20.00	3.21	1.30	0.51	2.48	0.39
50.00	8.20	1.70	0.91	7.47	0.87
132.00	22.22	2.12	1.35	21.50	1.33

## 6.5. PROPOSED MODEL BASED ON THE OBSERVED KINETICS

Initial interpretation of the observed behaviour involved the proposal of the following set of 'minimum assumptions' illustrated schematically in Fig. 6.7.

- 1) Helix formation is initiated at a metastable hairpin turn (for example a  $\beta$ -bend) which brings two chain segments into close spatial proximity.
- 2) These may then form the nucleus for a triple helix by collision with a third strand.
- 3) At low concentration the third strand is most likely to be a segment of the same chain, while at higher concentrations an intermolecular collision is more probable.
- 4) Nucleation is rate limiting, so that the intramolecular process shows first order kinetics and the intermolecular process second order.

These proposals therefore seem to explain the observed kinetic data. As mentioned previously (Chapter 1), proline and hydroxyproline are particularly effective in promoting formation of  $\beta$  bends and related tight turns; thus the likelihood of  $\beta$  bends being present in random coil gelatin as potential helix nucleation sites is a realistic possibility. Furthermore, this model offers a

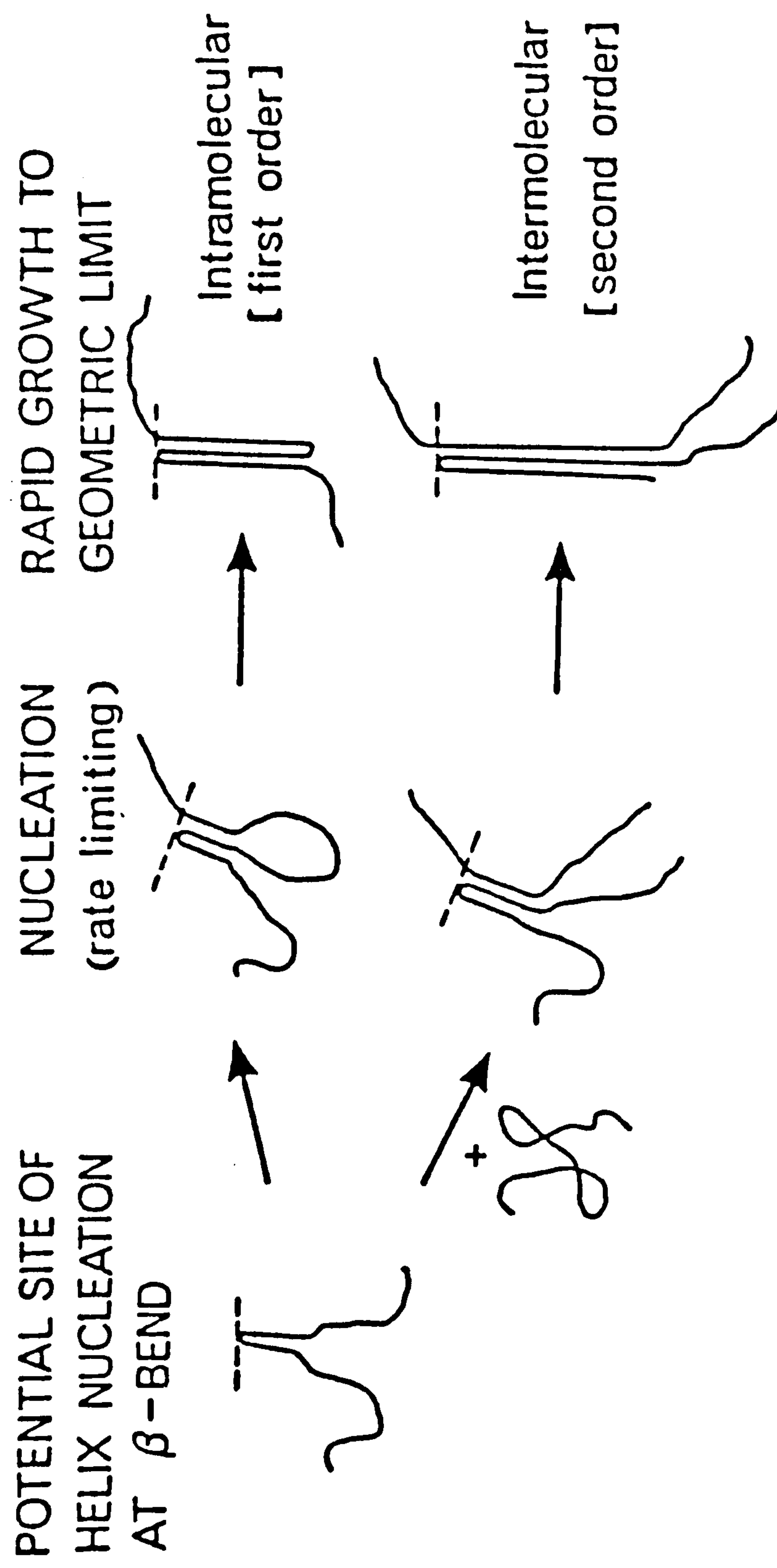


Figure 6.7 Schematic illustration of the proposed kinetic model, with the additional assumption (see Chapter 7) that propagation proceeds to the geometric limits.



simple explanation of the increased rigidity of gels whose thermal history has included a period of time at higher temperature. Since intramolecular helices involve three strands from the same chain, their maximum possible length will be one third of the chainlength, while for intermolecular helices, where only two of the participating strands are from the same chain, the maximum helix length will be half the chainlength. It would therefore be expected that the requirement for progressively longer helices with increasing temperature of renaturation would favour the formation of intermolecular helices (which will of course contribute to the strength of the gel network) in preference to shorter, intramolecular helices (which are 'wasted' in terms of network formation).

Increasing gelatin concentration not only increases the rate of reaction but also alters the shapes of the melting profiles obtained from DSC. Fig. 6.8 shows the melting endotherms after 16 hours at 5°C for gelatin samples of concentrations 2 mg ml<sup>-1</sup>, 20 mg ml<sup>-1</sup>, and 200 mg ml<sup>-1</sup>, superimposed on the same plot. As the gelatin concentration increases the shape changes and, in particular, the width of the endothermic peak increases. This effect can also be explained in terms of the model presented above. Longer helices, formed preferentially by the intermolecular process which is favoured at higher concentrations, would be more stable and therefore melt at higher temperatures than the

shorter intramolecular helices favoured at lower concentrations.

The peaks also broaden at lower temperatures for higher concentrations, indicating an increased number of shorter, less stable, structures as well. This unexpected result can again be interpreted qualitatively on the basis of the proposed reaction scheme. After initial helix formation, the residual loops and tails of the gelatin chains will be much shorter than the original chains (Fig. 6.7). The maximum helix length for intramolecular structures is one third of the sequence length, whereas for intermolecular structures it is half the sequence length (for hairpin elements of the triple helix) or the full sequence length (if the sequence is the third strand in the intermolecular association). Thus the intermolecular process favoured at higher concentrations can accommodate the short sequences into helical structures more easily than the intramolecular process dominant at lower concentrations, with consequent increase in the population of short, low-melting helices, as observed.

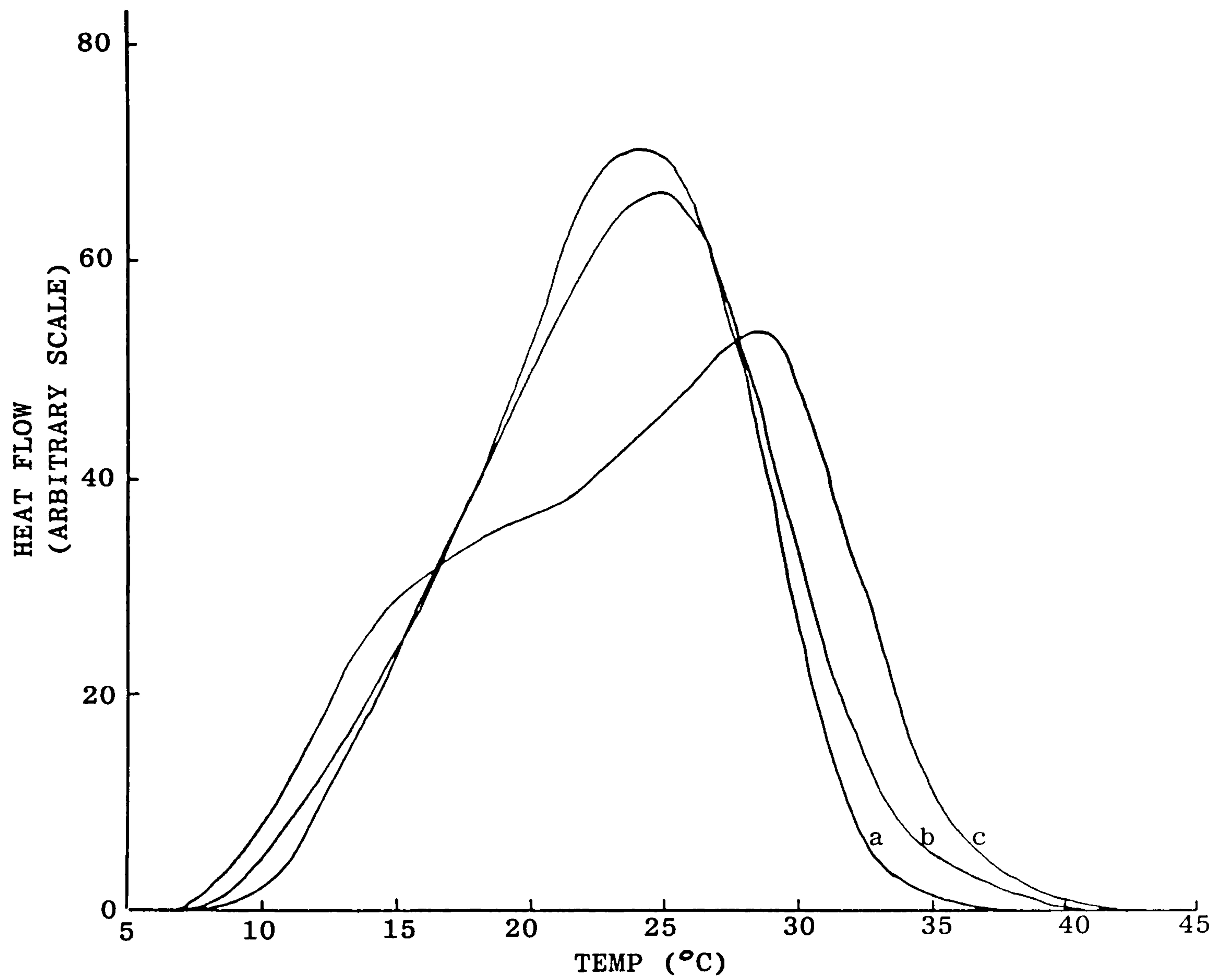


Figure 6.8 DSC melting profiles of LO-1 samples having differing concentrations a) 2 mg ml<sup>-1</sup>; b) 20 mg ml<sup>-1</sup> and c) 200 mg ml<sup>-1</sup> but normalised for comparison. Each sample had been quenched to 5°C for 16 hours prior to melting.



## 6.6. CONCLUDING REMARKS

From this study of the initial rate of conformational ordering for sample LO-1, a simple set of qualitative assumptions have emerged which will explain both the kinetic observations and, to a certain extent, the melting behaviour of the gelatin sample. The kinetic results are, however, restricted to the initial period of the reaction and tell us little about the obviously more complex behaviour occurring during the later development of helix structure in the logarithmic stage. In the next chapter, the proposed model is used as a starting point for the development of a more quantitative, unified analysis of gelatin renaturation and melting processes by Monte Carlo simulation.

## CHAPTER 7: MONTE CARLO SIMULATION OF GELATION AND MELTING

7.1. INTRODUCTION

Various theoretical treatments of gelatin gelation have been discussed in the Introduction to Chapter 6 but in spite of the fact that limited success has been achieved (by means of various analytical expressions), there still remains no overall model which can be used to match and predict all the observed experimental behaviour. The rate of helix formation, particularly in the latter stages of renaturation, has proved extremely difficult to simulate. Although Djaborouv *et al.* (1983, 1985) have managed to obtain good agreement between measured and calculated optical rotation values, their calculated values have involved the use of phenomenological expressions which shed little light on the underlying changes in molecular organisation. The complex behaviour at longer times must result from the involvement of various processes (e.g. helix winding, helix unwinding, helix nucleation, etc.) occurring simultaneously, but at different rates, during renaturation. Consequently, any kinetic analytical expression based on such events is bound to reflect this complexity and may ultimately be too difficult to deconvolute in terms of simple, identifiable molecular processes.

As a first step in overcoming these analytical difficulties, the hypothesis suggested at the end of Chapter 6 has been tested by the development of a "Monte Carlo" computer simulation program, using the assumptions as a model, to simulate the multiple molecular events likely to occur within a gelatin solution on cooling. The principle behind the Monte Carlo method, as the name suggests, is to treat the system as a "game of chance" in which simple molecular events can be considered in series. The likelihood of a particular event occurring is determined using the basic assumptions, whilst also taking into account the effects that any previous events may have had on the system. This type of treatment is particularly suited to computerisation as it involves numerous sequential calculations, the results of which can then be represented in terms of the entire system at any time by equating real time to the number of calculations performed.

The remainder of this chapter details the development of a gelatin simulation program (see Appendix 1 for final version) and presents the results computed, with particular reference to the effect that different starting parameters can have on the simulated renaturation and melting curves.



## 7.2. DESCRIPTION OF SIMULATION PROGRAM

As a first attempt at reproducing the experimentally observed optical rotation data, a computer program was developed incorporating the four assumptions arising from the kinetic analysis in Chapter 6 along with the additional assumption that subsequent helix growth is rapid and proceeds to the geometric limits, which would be reasonable if nucleation is the rate-limiting step.

The first phase of program development to implement and test these postulates will now be discussed in further detail with emphasis on the nature of the adjustable parameters and algorithms used. First of all, a large population of chains is generated, with a Gaussian length distribution centred around a chosen fraction of the maximum chainlength (i.e. the length of an intact a chain). The precise number of chains is a program variable as is the probability  $[p(\text{inter})]$  that helix formation will occur intermolecularly rather than intramolecularly. In each cycle of the program a chain (or, as discussed below, a chain segment) is selected at random (with the probability of selection being directly proportional to its length). The type of ordering to which the chain will be subjected (i.e. intermolecular or intramolecular helix formation) is then determined by comparing  $p(\text{inter})$  to a randomly generated number ( $p$ ) between 0 and 1. If  $p(\text{inter})$  is greater than  $p$  then intermolecular helix formation is considered and if

$p(\text{inter})$  is less than  $p$ , intramolecular ordering is simulated.

If the selected chain (or chain segment) is disordered (as of course it would be at the start of the simulation) then a point within it is chosen at random to simulate the position of the metastable 'hairpin bend' shown in Fig. 6.7. A second point is then chosen at random, either within the same sequence or within a different disordered sequence, according to the result of the ' $p(\text{inter})$  test' described above. This second point is regarded as the position at which the third strand joins the metastable turn to form the helix nucleus (Fig. 6.7). The program then calculates the maximum possible length of the helix, assuming propagation to geometric limits. The various geometric possibilities for different relative positions of the metastable turn (X) and the point on the third strand (Y) are illustrated in Figs. 7.1 and 7.2 for intramolecular and intermolecular nucleation, respectively. Also shown is the manner in which the various ordered and disordered chain sequence lengths were calculated.

Once the potential helix length has been calculated, the stability of the helix is assessed by determining the probability of unwinding,  $p(\text{un})$  (see Section 7.3) and again testing against a random number between 0 and 1.

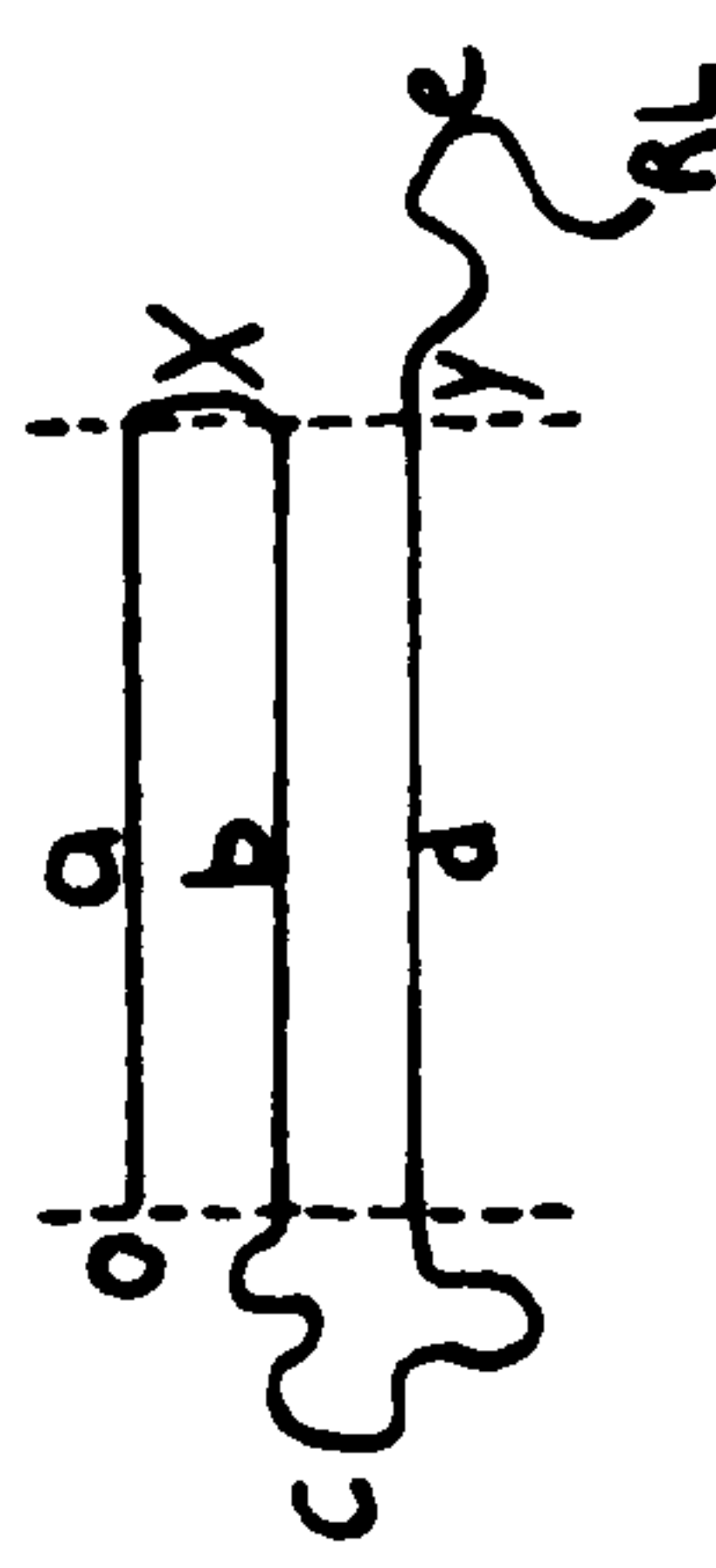
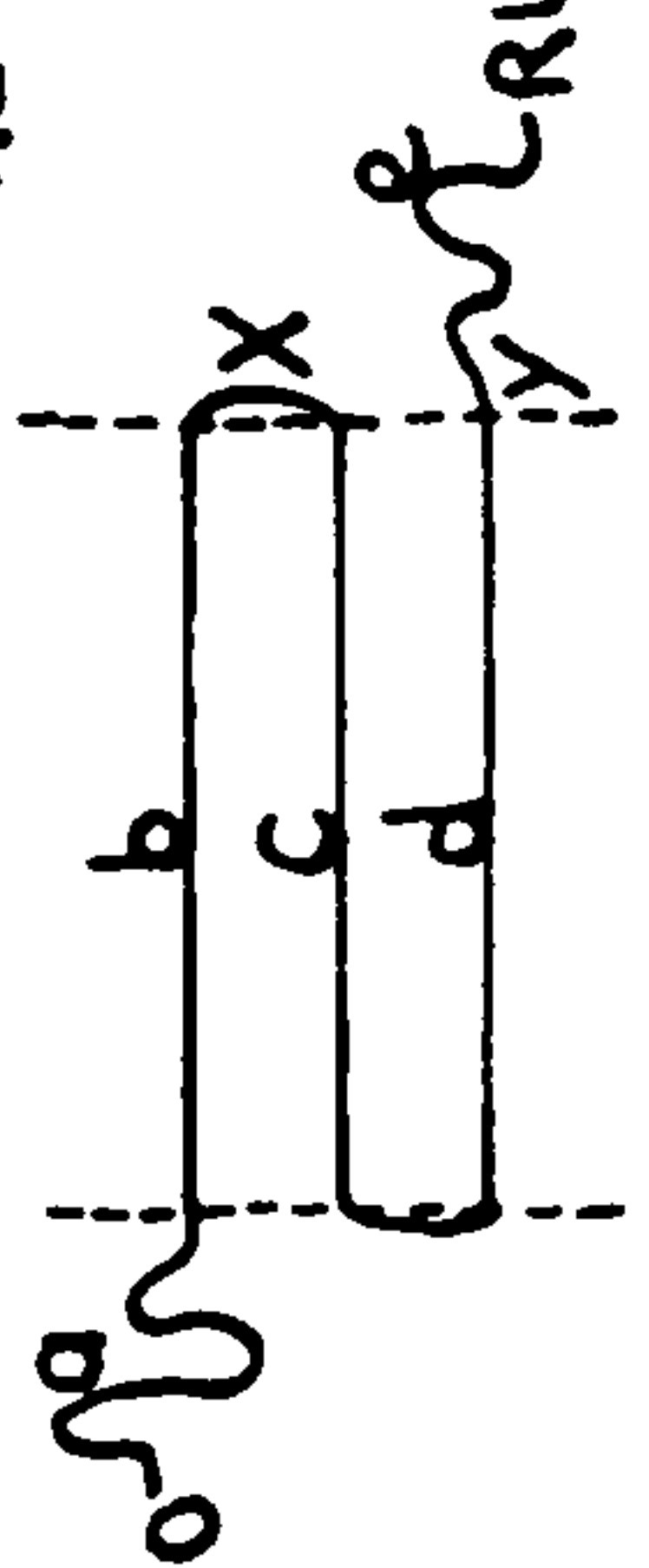
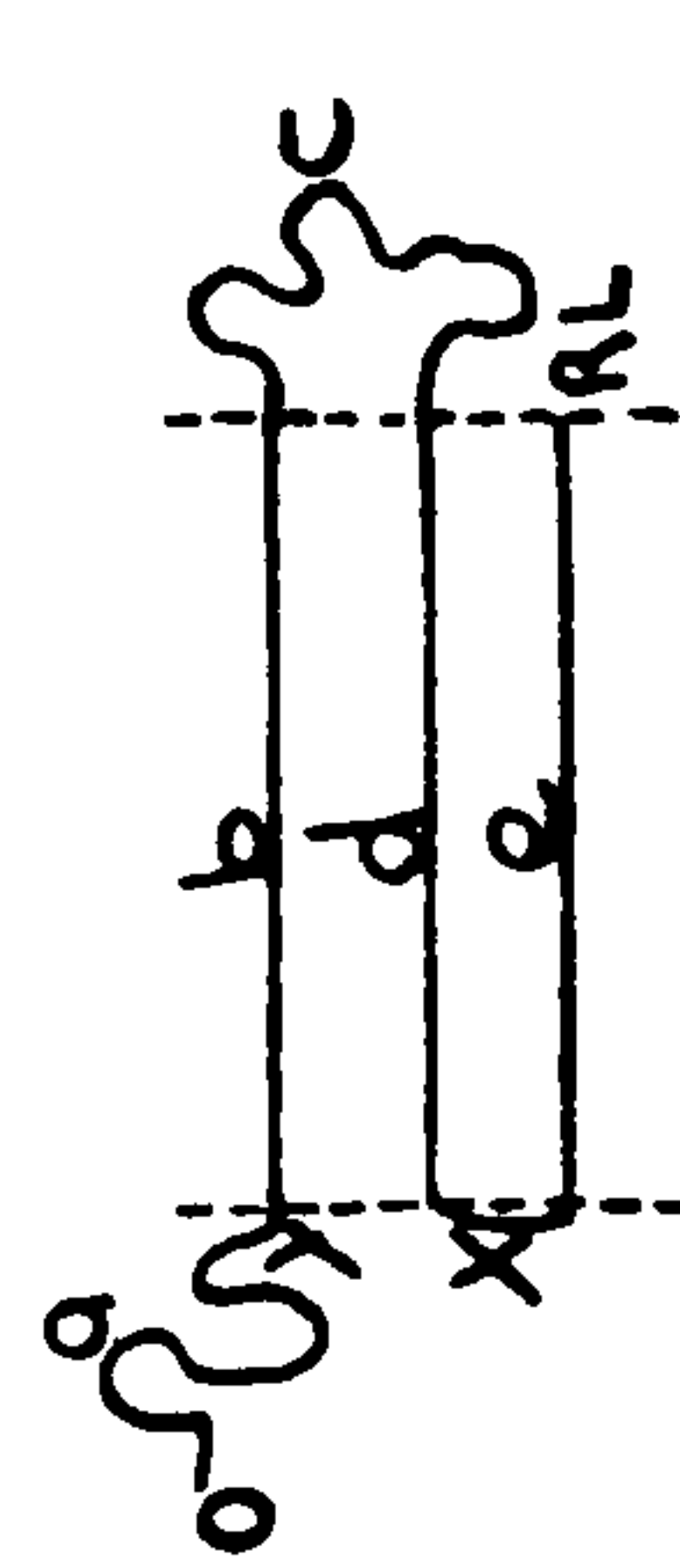
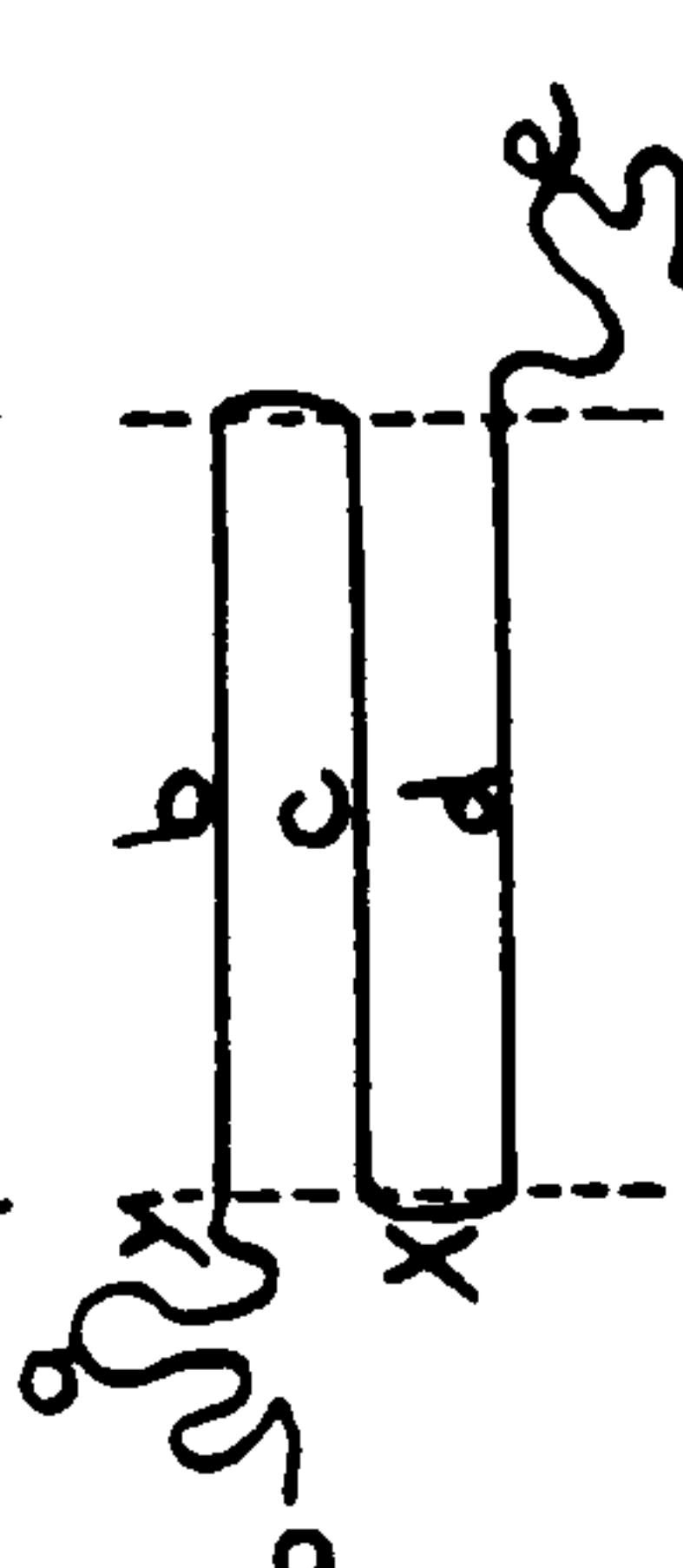

CASE	CONDITION		HELIX LENGTH	$\frac{a}{b}$	SEQUENCE LENGTH	$\frac{d}{e}$
1	$X < Y$ $X < \frac{Y-X}{2}$		$X$	$X$	$Y-3X$	$X$ $RL-Y$
2	$X < Y$ $X > \frac{Y-X}{2}$		$\frac{Y-X}{2}$	$\frac{3X-Y}{2}$	$\frac{Y-X}{2}$	$\frac{Y-X}{2}$ $RL-Y$
3	$X > Y$ $RL-X < \frac{X-Y}{2}$		$RL-X$	$Y$	$3X-2RL-Y$	$RL-X$
4	$X > Y$ $RL-X > \frac{X-Y}{2}$		$\frac{X-Y}{2}$	$Y$	$\frac{X-Y}{2}$	$\frac{X-Y}{2}$ $RL-(3X-Y)/2$

Figure 7.1. The four cases in intramolecular nucleation for the positions of the metastable turn (X) and the point on the third strand (Y) when considering propagation to continue to the geometric limits. In all four cases, the chain is split into three ordered sequences and two disordered sequences, the lengths of which are shown for each case along with the helix length. RL represents the length to the end of the chain.



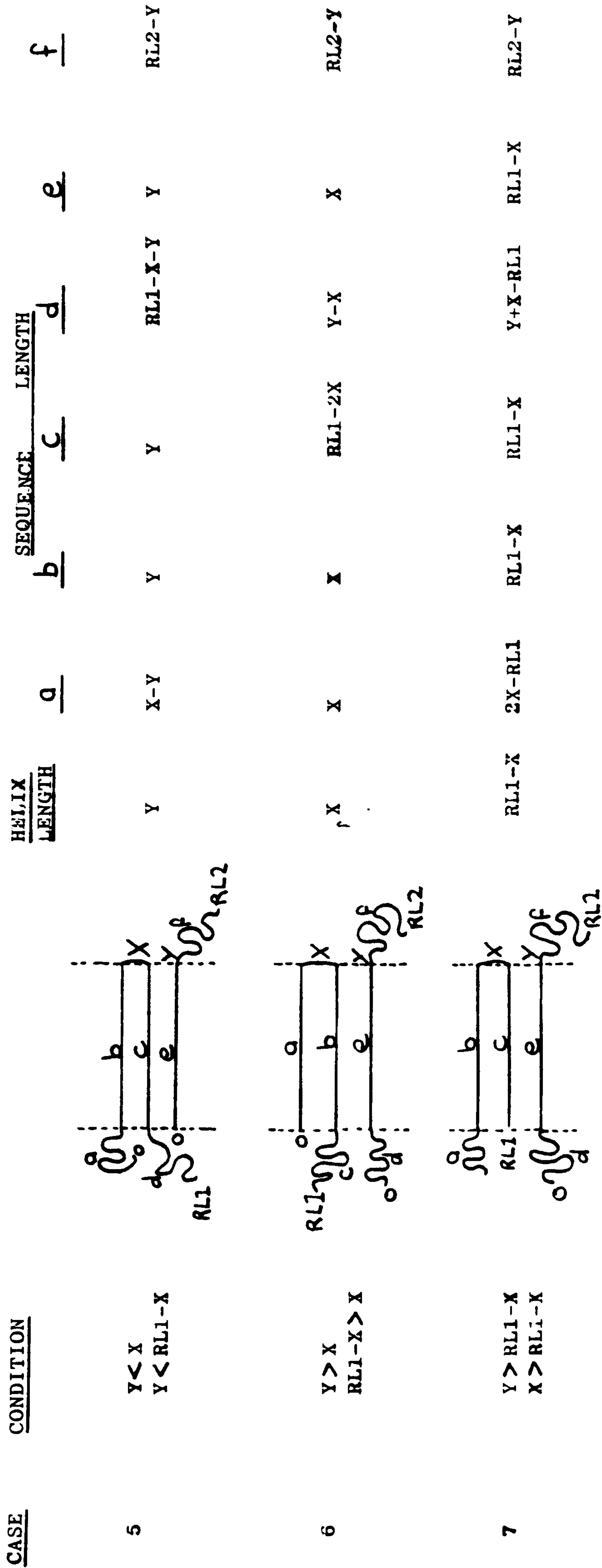


Figure 7.2. The three cases in intermolecular nucleation for the positions of the metastable turn (X) and the point on the third strand (Y) when considering propagation to continue to the geometric limits. The chains are split into three ordered and three disordered sequences in all three cases, the lengths of which are shown for each case along with the helix length. RL1 and RL2 represent the lengths to the end of the chains for the chain containing the metastable turn and the point in the second chain, respectively.

If  $p(un)$  exceeds the random number, nucleation is considered to have failed and the program continues to the next cycle, leaving the chain(s) disordered. Otherwise the three participating strands of the helix are stored as ordered sequences, and any residual disordered loops or tails are considered as additional candidate sequences for helix formation in later cycles of the program.

The discussion so far has considered only the case in which the randomly selected sequence is disordered. If, however, it is already ordered (which becomes progressively more likely as the simulation proceeds) then the stability of the ordered structure is tested as above. If  $p(un)$  is less than the random number generated, the program continues on to the next cycle leaving the helix intact; otherwise the chosen sequence and its two helix partners are considered to have become disordered and are combined with any contiguous disordered sequences in the same chain(s).

The program periodically tabulates helix fraction and average helix length for both inter- and intra-molecular structures. The interval (i.e. number of iterations) between table entries is selected at the start of execution, along with the desired total number of iterations. When this has been completed the user has the option of printing out various sets of data relating to the simulation before either altering the program

variables, continuing to a larger number of iterations with the same program variables, or stopping. The printing options available include a table giving the number and length-distribution of inter- and intra-molecular helices, a simulated melting profile where residual helix-fraction is tabulated as a function of increasing temperature, and a table of chain sequences in which all the sequences are listed, with (in the case of ordered sequences) the locations of their helix partners (primarily for diagnostic purposes during program development).

### 7.3. HELIX STABILITY TEST

In the last section it was mentioned that the stability of any particular helix was assessed by determining the probability of unwinding,  $p(\text{un})$ , and testing this against a random number between 0 and 1. Initially, the method used to obtain  $p(\text{un})$  involved an exponential function (Equation 7.1)

$$p(\text{un}) = A \exp(-BL) \quad (\text{Equation 7.1})$$

in which  $L$  is the helix length and  $A$  and  $B$  are constants with preselected values chosen to simulate temperature. Using such stability criteria it was possible to simulate renaturation curves not dissimilar from those obtained experimentally and helix length distributions



with bimodal character (see Busnel *et al.*, 1987; Appendix 2). The exponential approach, however, was by no means satisfactory as the equation and variables have no theoretical basis, the values of A and B producing the best fits being purely empirical. The next stage was therefore to develop an alternative approach using program variables with direct physical significance.

A central concept in coil-helix transition theory is the initial formation of a helix nucleus, followed by a rapid propagation step with a competing back-reaction ('zipping and unzipping'). The interplay between these processes has been the subject of numerous papers (as reviewed by, for example, Poland & Scheraga, 1970; Cantor & Schimmel, 1980), and gives rise to highly complex kinetic equations which in many cases have no analytical solution. Within the framework of the Monte-Carlo simulation, however, such complexity is avoided by simulating the forward and back reactions individually in different cycles of the program, and treating each as a simple two-state all-or-none process. Using this approach, the thermal stability of the helical structure and the overall kinetics of helix formation can both be analysed in terms of helix length and the difference in free energy between the ordered and disordered states ( $\Delta G$ ).

At the transition midpoint temperature ( $T_m$ ),  $\Delta G$  is zero

$$\text{i.e. } \Delta G = \Delta H - T_m \Delta S = 0 \quad (\text{Equation 7.2})$$

Thus the entropy change ( $\Delta S$ ) is related to the corresponding change in enthalpy ( $\Delta H$ ) by:

$$\Delta S = \Delta H / T_m \quad (\text{Equation 7.3})$$

For a helix of  $n$  residues (or, in the case of gelatin,  $n$  'units' comprising a residue from each of the three strands), the overall value of  $\Delta S$  is equal to  $n\Delta S_r$ , where  $\Delta S_r$  is the entropy loss per unit. As discussed in Chapter 1, however, the overall change in enthalpy is  $(n-x)\Delta H_r$ , where  $\Delta H_r$  is the enthalpy gain on addition of a further unit to the helix (and is equal to the experimental value,  $\Delta H_{cal}$ , from DSC) and  $x$  is the number of units which cannot participate in bonding due to end effects. The melting temperature of the helix is therefore related to its length by:

$$T_m = \Delta H / \Delta S = (n-x)\Delta H_r / n\Delta S_r = T_m' (n-x)/n \quad (\text{Equation 7.4})$$

where  $T_m'$  is the value of  $T_m$  for a helix of infinite length (i.e. as  $(n-x)/n \rightarrow 1$ ).

The value of  $x$  (which controls the co-operativity of the transition) is specified directly at the start of the program and  $T_m'$  (reflecting the balance between entropy loss and enthalpy gain on addition of a unit to the helix) is defined by an estimated value for the melting point of native collagen ( $T_c$ ). Taking the number of residues per a chain as 1000,  $T_c$  can be converted (Equation 7.4) to the corresponding value of  $T_m'$  for the current value of  $x$  by:

$$T_m' = 1000 T_c / (1000 - x) \quad (\text{Equation 7.5})$$

The thermal stability for each helix length ( $n$ ) can then be defined by the equilibrium constant ( $K$ ) between the disordered and ordered states, using the standard relationship:

$$\Delta G = RT \ln K \quad (\text{Equation 7.6})$$

where  $R$  is the gas constant. (The usual negative sign in this equation is omitted as  $\Delta G$  is defined in terms of the forward reaction, i.e. helix-coil and  $K$  in terms of the reverse reaction i.e. coil-helix). From Equations 7.2 and 7.3:

$$\Delta G = \Delta H - T\Delta S = \Delta H - T\Delta H/T_m = \Delta H(T_m - T)/T_m \quad (\text{Equation 7.7})$$

$$\text{Thus } \ln K = n\Delta H_{cal} (T_m - T)/RT T_m \quad (\text{Equation 7.8})$$



K is calculated using the value of  $T_m$  for each  $n$ , derived as described above (Equation 7.4), and the enthalpy change per helical unit ( $\Delta H_{cal}$ ), which is specified at the start of the program.

The probability of unwinding,  $p(un)$ , can be defined as the proportion of residues in the disordered form at temperature  $T$ , and is therefore related to  $K$  by the simple expression:

$$p(un) = 1.0/(K + 1.0) \quad \text{(Equation 7.9)}$$

As described in the previous section, this stability test is applied (by comparison of  $p(un)$  with a random number between 0 and 1) to decide whether a new helical sequence is sufficiently long to be stable at the current temperature and whether or not existing helical sequences will be unwound. The section of the program which determines the melting profile utilizes the same equations to determine residual helix fraction as a function of temperature.

The results obtained using the program with the five initial assumptions and the stability test described above, will now be illustrated and discussed.

#### 7.4. INITIAL RESULTS FROM THE SIMULATION PROGRAM

The adjustable parameters discussed above are tabulated in Table 7.1 along with the corresponding names for these variables in the Fortran listing (Appendix 1) and typical values used when running the program. The calorimetric enthalpy and melting point of collagen should ideally be fixed at the appropriate values for the gelatin whose behaviour is being simulated. However, such precise data is not available and so these two parameters were varied within realistic limits (see Table 7.1). The maximum number of iterations has to be large enough to allow each chain to be randomly selected several times if the effects of annealing, in the later stages of renaturation, are to be simulated.

The results obtained under typical running conditions are illustrated in Fig. 7.3 as a plot of helix fraction versus number of iterations for simulated quench temperatures of 25°C and 5°C; the details of the other parameters used are indicated on the plot. The graph shows what was found to be a common trend for all variations of the parameters, namely, that the helix fraction obtained at high temperature was almost as great as that obtained at low temperature, which is clearly inconsistent with the experimental data.



The inadequacies of the initial assumptions are further demonstrated by the simulated melting profiles. As shown in Fig. 7.4, the maxima in the profiles obtained for simulated quench temperatures of 5°C and 25°C are both at virtually the same position, with only slight skewing to lower melting temperatures for the much lower quench temperature, again completely at variance with experimental behaviour.

An alternative way of envisaging these results is that, at both temperatures, large populations of long helices are formed which are then stable to high temperatures, thereby eliminating the chance of the formation of short helices which would have lower melting temperatures.

These simulated results were unexpected and posed the question of why such long ordered sequences should be formed, even at low temperatures. The answer was, of course, that propagation to geometric limits allows the formation of long stable helices, regardless of the quench temperature. The result of this effect was that the program did not allow any significant annealing-in of longer helices with increased holding temperature as envisaged. In order to overcome this problem, the assumption that propagation continued to the geometric limits had to be rejected and replaced by some other criterion which stopped or delayed propagation, thereby allowing shorter helices to form. The program modification and the theoretical basis of the new assumptions is discussed in the next section.



Table 7.1. Adjustable parameters used in the simulation program, with their corresponding names in the Fortran listing and typical values used.

PARAMETER	FORTRAN NAME	TYPICAL VALUES USED
NUMBER OF CHAINS	NCT	100-1000
NUMBER OF ITERATIONS	MAXIT	100-300000
ITERATION INTERVAL	NTAB	SUITABLE FRACTION OF MAXIT
p(inter)	PINTER	0-100 (USUALLY 10)
$T_c$	TMAX	36-40°C
$\Delta H_{cal}$	DHCAL	12-16 kJ/mol
x	XNUC	1-4
CHAINLENGTH DISTRIBUTION	RLMIN	0-1
SIMULATION TEMPERATURE	TC	5-30°C

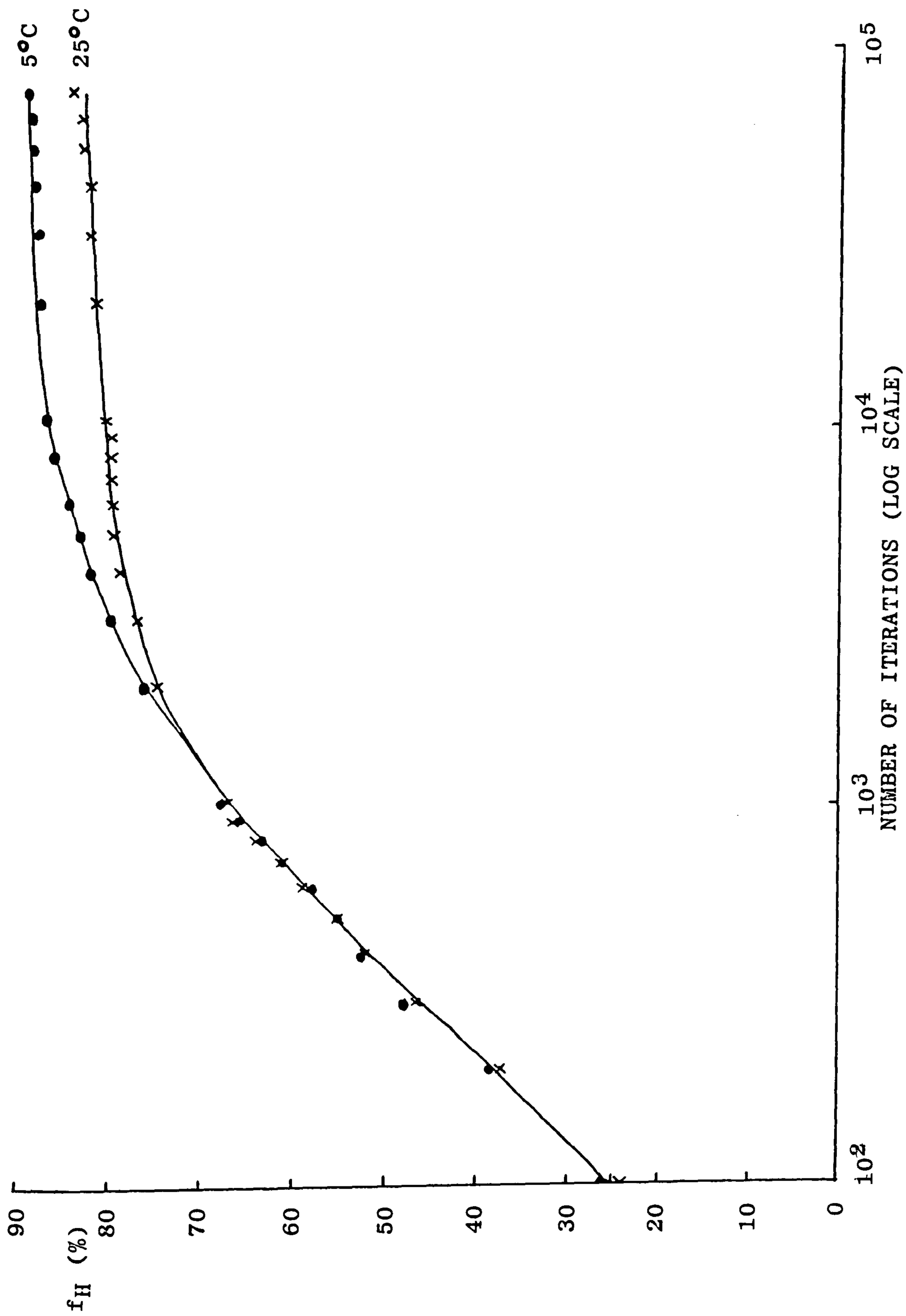


Figure 7.3. Simulated helix fractions versus the number of iterations for quench temperatures of 25°C (X) and 5°C (●) using the assumption that propagation proceeds to the geometric limits. Other program parameters are  $x = 1$ ,  $T_c = 38^\circ\text{C}$ ,  $p(\text{inter}) = 10$ ,  $\Delta H_{cal} = 12.8$ , no. of cycles = 300,000, no. of chains = 200.

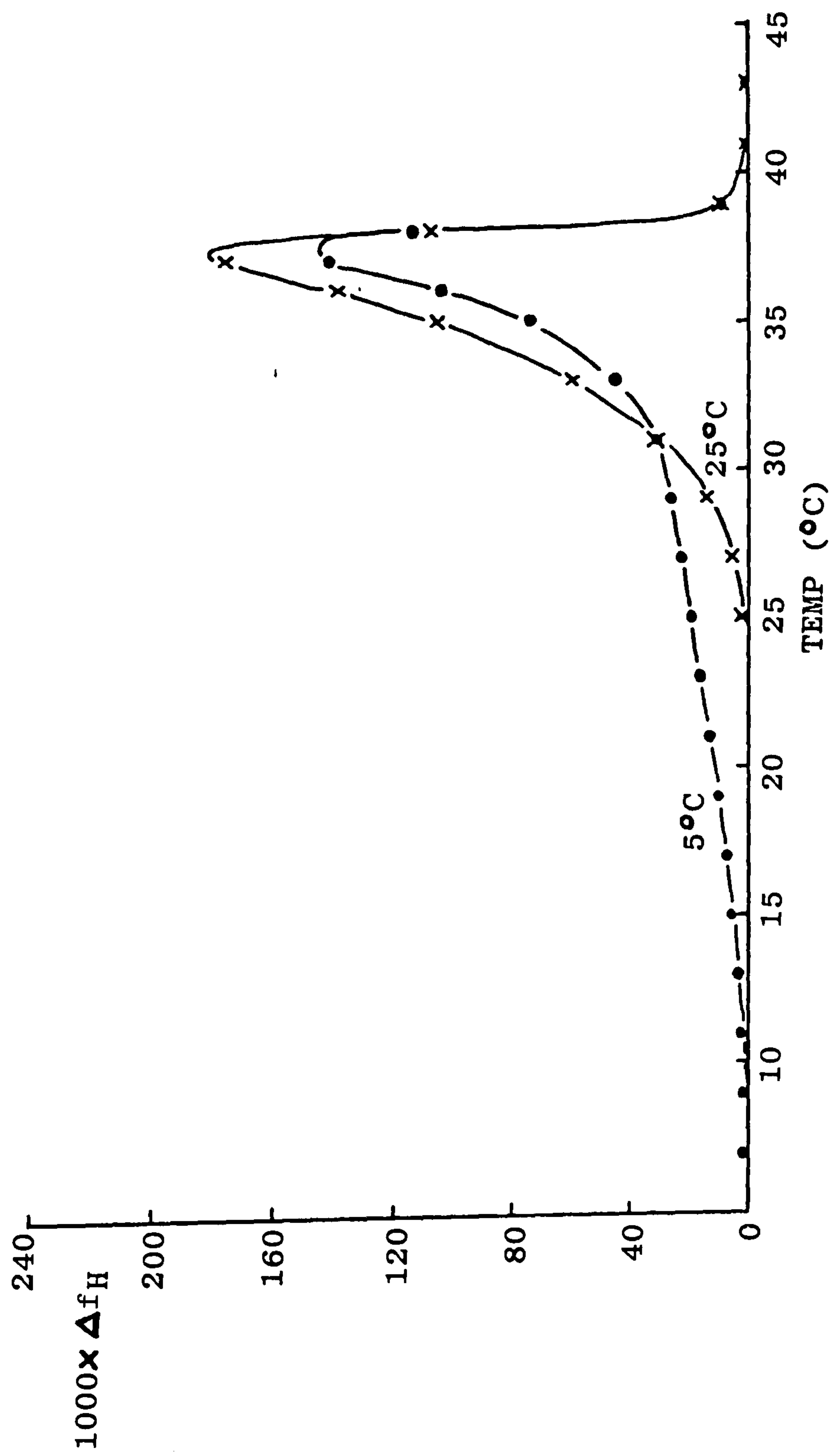


Figure 7.4. Simulated melting profiles for quench temperatures of 25°C (X) and 5°C (●), plotted in terms of the change in helix fraction ( $\Delta f_H$ ) as a function of temperature for simulations where propagation to geometric limits has been assumed. Other program parameters are the same as in Fig. 7.3.



### 7.5. HELIX SIZE LIMITATIONS

As discussed in Chapter 1, the energy differences between the *cis* and *trans* forms of imino residues are much smaller than in other amino acids, due to the secondary amine structure. This lower energy difference causes a larger proportion of the residues to exist in the *cis* configuration, the geometry of which is incompatible with ordered packing into the collagen triple helix structure. Isomerisation of these 'cis kinks' must therefore take place if helix propagation is to continue.

Studies of the renaturation of collagen samples in which the three strands are linked together covalently, thus eliminating the nucleation step in helix formation, indicate that the *cis-trans* isomerisation process can dominate the overall rate of helix propagation (Bächinger *et al.*, 1978, 1980). The renaturation process shows two distinct phases: a very rapid phase corresponding to 'zipping up' of helices to the first *cis* 'kink' followed by a much slower process with a rate constant closely similar to those measured for *cis-trans* isomerisation in model peptides (Cheng and Bovey, 1977). For collagen fragments with short, truncated chains (which have a low probability of including an imino residue in the *cis* form) the rapid phase is dominant, but renaturation of intact collagen follows the kinetics of the slow isomerisation process over virtually the entire transition.

A mechanism involving such behaviour would be capable of producing the shorter, less stable helices required in the simulation program. On reaching a *cis* peptide group propagation would effectively stop while *cis-trans* isomerisation was occurring, and during this period further nucleation within the same chain(s) could take place, thereby reducing the effective maximum helix length attainable when the *cis-trans* barrier was surmounted. Alternatively the shorter triple helix sequence could be unstable and unwind before the *cis-trans* barrier was overcome.

Incorporation of the *cis-trans* isomerisation effect into the program was achieved by the use of two further program parameters: PKINK, the probability of the occurrence of a 'cis kink' and PTRANS, the likelihood of such a barrier being overcome. Consider propagation of a helix in terms of the three chain sequences involved. In order to increase the length by one unit, three residues (one from each chain sequence) must become ordered, and as the imino acids contribute approximately a third of the total composition, this means that, on average, an increase in helix length of one unit involves ordering one imino acid group. PKINK can therefore be regarded as the probability that the next imino residue to be ordered during propagation is in the *cis* configuration and (1-PKINK) as the probability that the next imino residue is in the *trans* configuration.



Thus the probability ( $p$ ) that all imino residues up to the  $n$ 'th imino residue added to the helix are in the *trans* form is given by:

$$p = (1 - \text{PKINK})^n \quad (\text{Equation 7.10})$$

which can be rewritten in logarithmic form as:

$$n = \log p / \log (1 - \text{PKINK}) \quad (\text{Equation 7.11})$$

The *cis-trans* isomerisation test was incorporated into the section of the program which calculates helix length. After deriving the maximum possible helix length using the 'geometric limits' criterion, the program proceeds by generating a random probability  $p$ , and then uses Equation 7.11 and the value of PKINK to determine  $n$ , which is considered as the helix length to the first 'cis kink'. The program then considers the probability of surmounting the *cis-trans* barrier by generating a random number between 0 and 1 and testing it against the value assigned to PTRANS. If PTRANS is greater than the random number it is assumed that the 'cis kink' has been overcome and the cycle involving PKINK is repeated to further increase the helix length until the PTRANS test finally fails.



If the 'cis-limited' helix length is greater than the 'geometrically-limited' helix length then the program continues as previously, using the geometric value. If, however, the cis-limited length is smaller (as is usually the case) it is then regarded as the length of helix formed. Figs. 7.5 and 7.6 show the way in which the disordered and ordered chain sequence lengths are calculated in the case of cis-limited helix formation for intra- and inter-molecular nucleation respectively.

PKINK is theoretically the equilibrium constant,  $K$ , between the *trans* and *cis* forms, and as such should ideally be fixed at a known, experimental value. However, as values of  $K$  varying between 0.03 and 0.666 have been quoted for model peptides containing proline and hydroxyproline (Bächinger *et al.*, 1980) PKINK has been varied within these limits during computer simulation. PTRANS, the probability of overcoming a 'cis kink', has also been varied and in real systems would obviously be related to the rate constant for *cis-trans* isomerisation. However, although PKINK and PTRANS are, in principle, completely unrelated parameters, on further consideration it has become evident that, within the simulation, the two are behaving mathematically in the same manner (i.e. restricting the length of the helix) and for this reason, in some of the later results presented, PTRANS has been set at zero and PKINK used as the only variable.

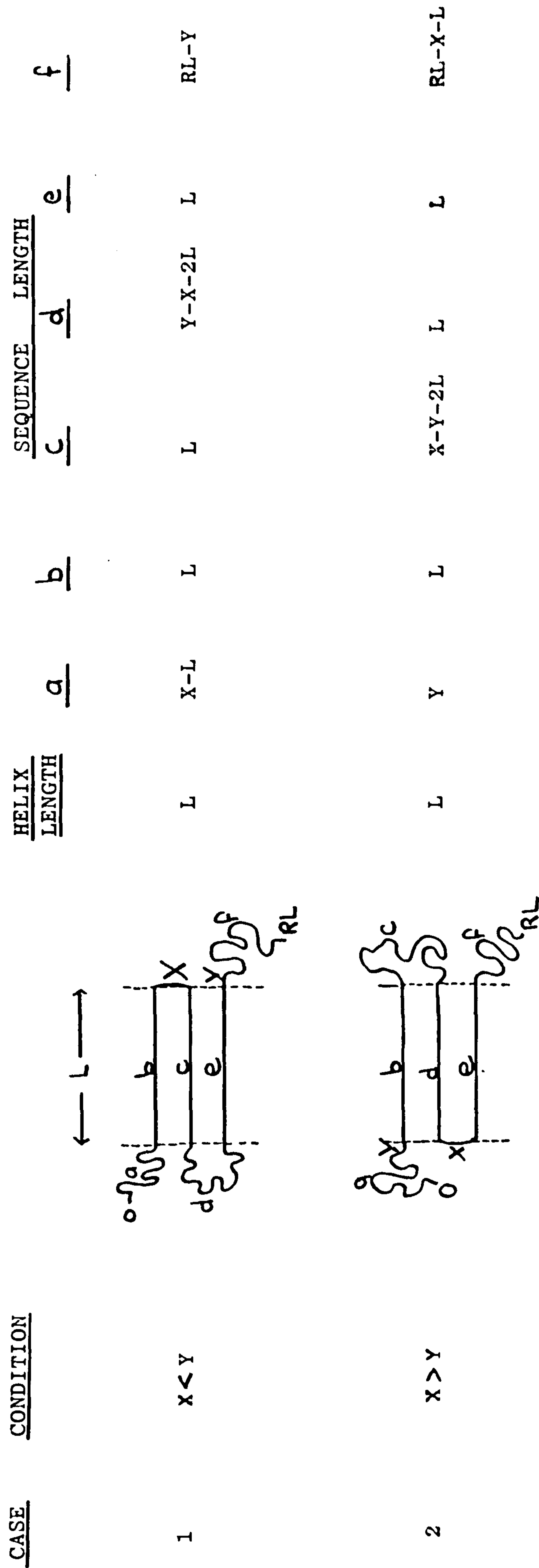
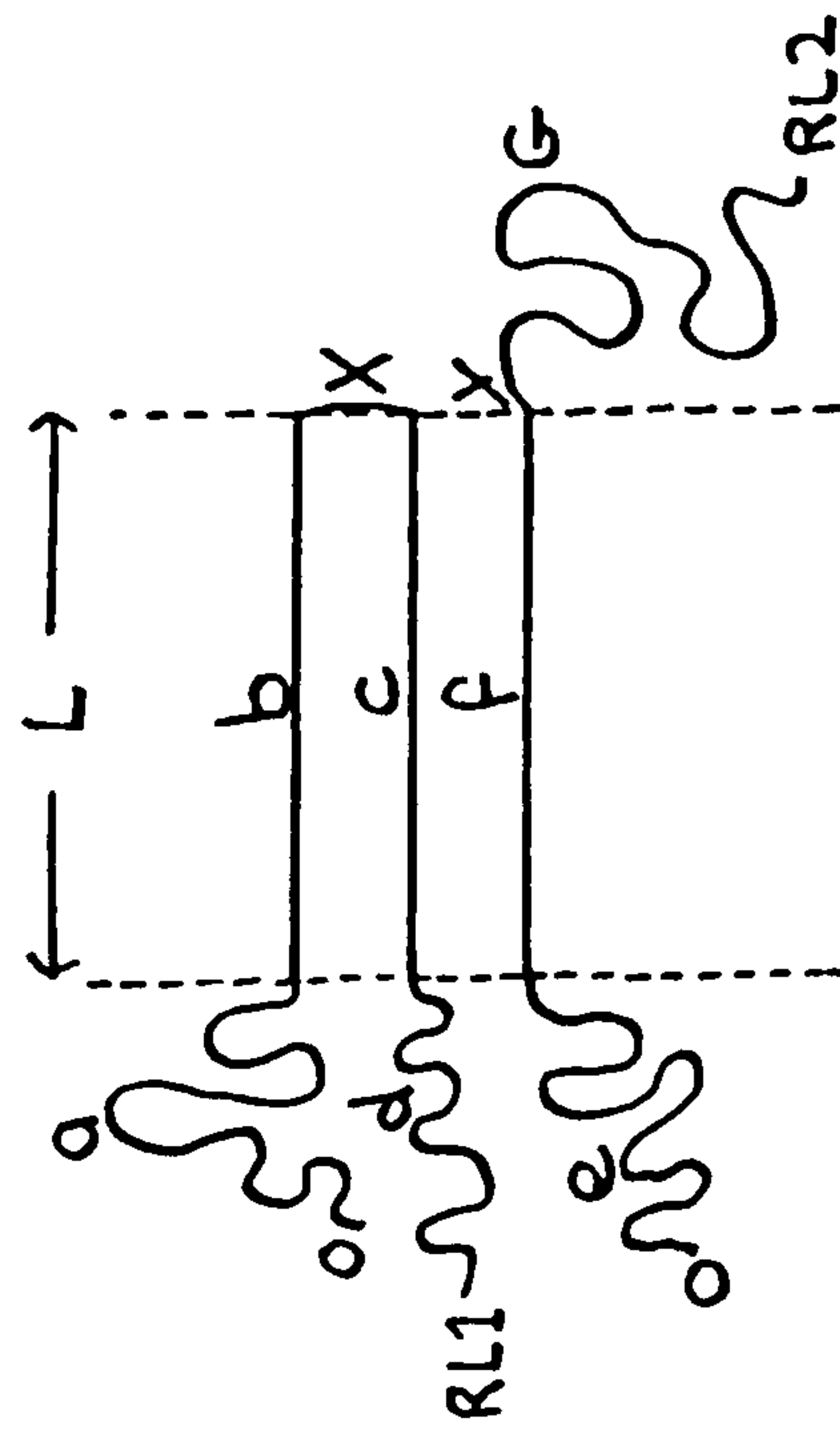


Figure 7.5. The two cases in 'cis-limited' intramolecular nucleation for the position of the metastable turn (X) and the point on the third strand (Y). Cases 1 and 2 and 3 and 4 in Fig. 7.1. degenerate to just two cases in 'cis-limited' nucleation ( $X > Y$ ) and ( $Y > X$ ). In both these cases three ordered and three disordered chain sequences are formed, the lengths of which are shown. RL represents the length to the end of the chain and L the length to the first insurmountable *cis* kink.



<u>HELIX</u>	<u>LENGTH</u>	<u>a</u>	<u>b</u>	<u>c</u>	<u>SEQUENCE</u>	<u>LENGTH</u>	<u>d</u>	<u>e</u>	<u>f</u>	<u>g</u>
L		X-L	L	L			RL1-X-L	Y-L	L	RL2-Y

Figure 7.6. The length of helix (L) and the lengths of the disordered and ordered sequences for 'cis-limited' intermolecular nucleation. The three cases in Fig. 7.2. degenerate to just one case in 'cis-limited' intermolecular nucleation. RL1 and RL2 represent the lengths to the ends of the chains for the chain containing the metastable turn and the point in the second chain, respectively.



Now that the program has been described in its entirety, the effect of varying all the different parameters will be discussed and simulation results compared with results obtained experimentally.

#### 7.6. EFFECT OF VARYING PARAMETERS

The parameters listed in Table 7.1 along with PKINK and PTRANS are the variables which will now be discussed in terms of the effect they have on simulated helix regeneration and melting profiles. Table 7.2 lists the 'target values' of experimental helix fractions and melting temperatures, obtained using a 20 mg ml<sup>-1</sup> LO-1 sample cured for a long period of time at, and melted out from, various holding temperatures.

##### 7.6.1. Parameters Having Minimal Effect on Simulations

The value used for the calorimetric enthalpy ( $\Delta H_{cal}$ ) was obtained from experimental DSC curves produced from melting gels of known total helix fraction (from optical rotation) and gelatin concentration. The enthalpy of the transition was calculated from the DSC melting profiles as discussed in Section 3.4 and converted into a value in kJ mol<sup>-1</sup> of residues using an average value of 100 for the molecular weight of an amino acid

residue. (The enthalpy change per residue was multiplied by 3, for simulation purposes, as the program deals in terms of the enthalpy change per tripeptide unit). Initial determinations of helix fractions and hence  $\Delta H_{cal}$  were later found to be slightly erroneous as the temperature-dependence of optical rotation in the sol state had not been taken into account during these calculations. Consequently, the value calculated for  $\Delta H_{cal}$  initially ( $12.8 \text{ kJ mol}^{-1}$ ) and used throughout the simulation runs was approximately  $2 \text{ kJ mol}^{-1}$  below the true value. However, for comparative purposes, the value of  $12.8 \text{ kJ mol}^{-1}$  has also been maintained in the majority of later simulations, particularly as it was shown (Fig. 7.7 a,b) that the effect of a small variation in  $\Delta H_{cal}$  has a negligible effect on the simulated renaturation and melting curves.

The variable  $p(\text{inter})$  was written into the program to enable simulation of different gelatin concentrations (by altering the relative proportions of inter- and intra-molecular nucleations) but, as illustrated in Fig. 7.8,  $p(\text{inter})$  has a negligible effect on the shape of the renaturation curves. This result is not surprising since, although  $p(\text{inter})$  was envisaged as a method of simulating concentration, this is only partially the case. In a real system a decrease in concentration must result in a decrease in the number of collisions causing helix nucleation; this effect will not be simulated by a decrease in  $p(\text{inter})$  which



dictates only the type of nucleation occurring, with the number of collisions (i.e. in simulation terms, the number of iterations) being the same for all values of  $p(\text{inter})$ . The value of  $p(\text{inter})$  does, to a certain extent, however, reflect the effect of increased gelatin concentrations on the experimental melting profiles of gelatin gels, as can be seen from Fig. 7.9 showing the simulated melting profiles after 300,000 iterations at 20°C for  $p(\text{inter})$  values of 0.1, 1 and 10. An increase in concentration favours the intermolecular process, which in turn should favour the development of more, larger, thermodynamically stable helices than the intramolecular process dominant at lower concentrations (as discussed in Section 6.5). It therefore follows that, particularly for higher renaturation temperatures, a larger proportion of helices melting at higher temperatures should be formed at high gelatin concentrations than at low gelatin concentrations. This anticipated behaviour is indeed observed, as an increase in size of the melting peak corresponding to the 20°C quench temperature, both for experimental curves in which concentration is increased (Fig. 7.10) and for simulated curves in which the value of  $p(\text{inter})$  is increased (Fig. 7.9).

Because much of the experimental work reported was performed on gels of concentration 20 mg ml<sup>-1</sup> (a concentration at which, according to Fig. 6.6, the intermolecular nucleation process is dominant)



$p(\text{inter}) = 10$  was estimated to be a reasonable value for the majority of attempts at simulating the experimental behaviour.

Another program parameter requiring some discussion is the number of chains in the simulation. This has to be large enough to give a statistically-reasonable representation of the gelatin molecules in solution. However, if too large, the time required to run the program becomes excessive, and consequently a compromise of between 100 and 1000 chains was generally used. The use of a smaller number of chains, while not giving a very accurate representation of the gelatin system, was often adopted during program development and testing to obtain sensible limits for the other program variables without the inconvenience of lengthy run times.

The final parameter to be considered in this section is the chainlength distribution. This should ideally be a true representation of the length distribution for the gelatin whose behaviour is being simulated. However, as such precise data was not available, a Gaussian chainlength distribution, centred around a chosen fraction of the length of an undegraded chain (in most cases one half) was utilised.

The parameters discussed above were program variables or experimental values obtained from the work presented previously which have only a minimal effect on the

Table 7.2. Target values of helix fractions ( $f_H$ ) and melting temperatures ( $T_m$ ) obtained from optical rotation measurements on a solution of LO-1 (20 mg ml<sup>-1</sup>) quenched to various holding temperatures ( $T_0$ ) and melted out.

$T_0$ (°C)	$f_H$ (%)	$T_m$ (°C)
5.0	58	22.0
15.0	47	25.5
20.0	36	28.1
25.0	18	31.1

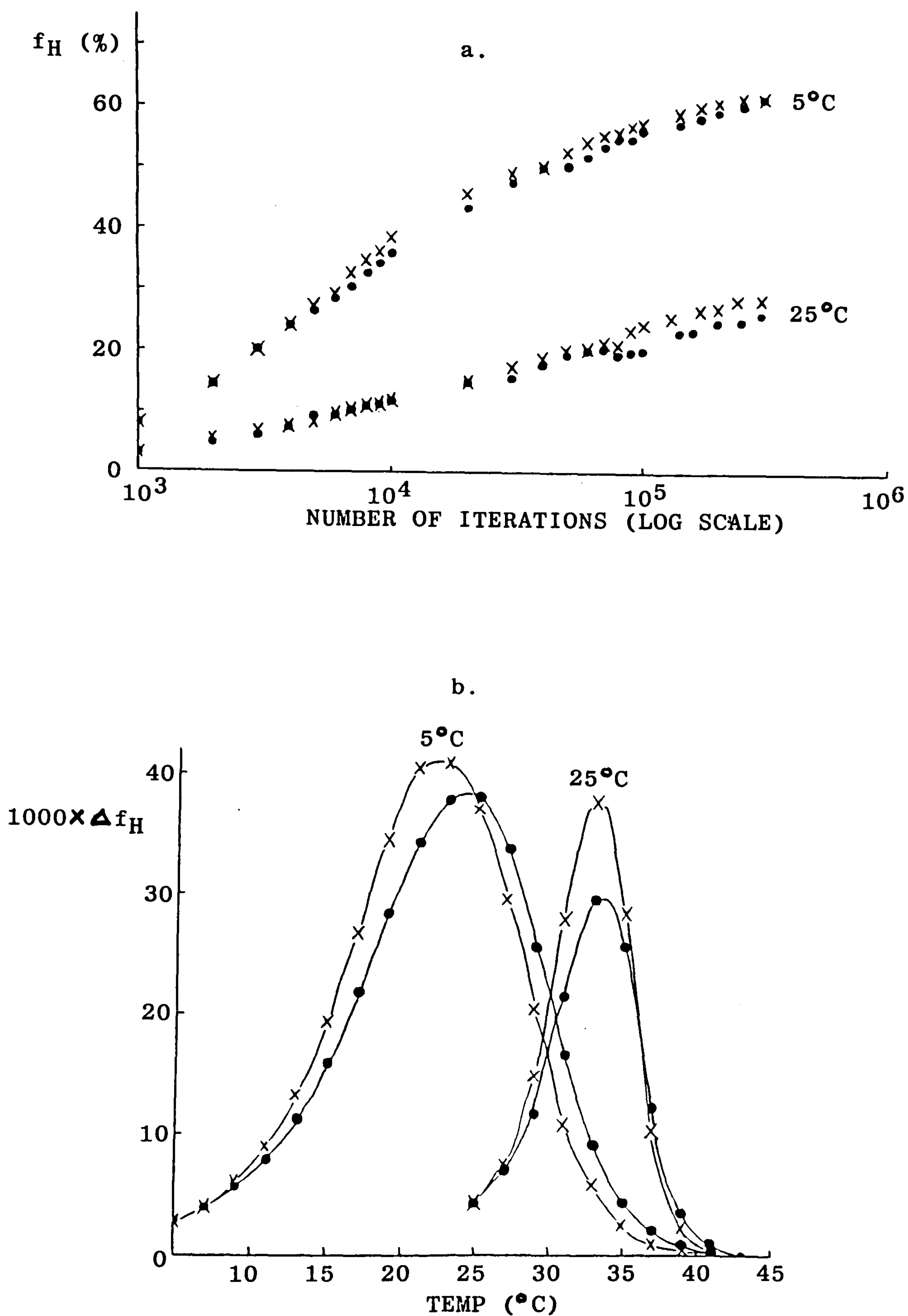


Figure 7.7. The effect of varying  $\Delta H_{cal}$  within experimental limits.

a) Renaturation curves at quench temperatures of 5 and 25°C using values of  $\Delta H_{cal}$  equal to 12.8 (●) and 15 (X) kJ mol<sup>-1</sup>. Other program parameters are:

- 1) For the 5°C curves:  $x = 1$ ,  $T_c = 38^\circ\text{C}$ ,  $p(\text{inter}) = 10$ , no. of cycles = 300,000, no. of chains = 200, PKINK = 0.15, PTRANS = 0.
- 2) For the 25°C curves:  $x = 1$ ,  $T_c = 40^\circ\text{C}$ ,  $p(\text{inter}) = 10$ , no. of cycles = 300,000, no. of chains = 200, PKINK = 0.15, PTRANS = 0.

b) Melting profiles of the simulated helices produced in a).



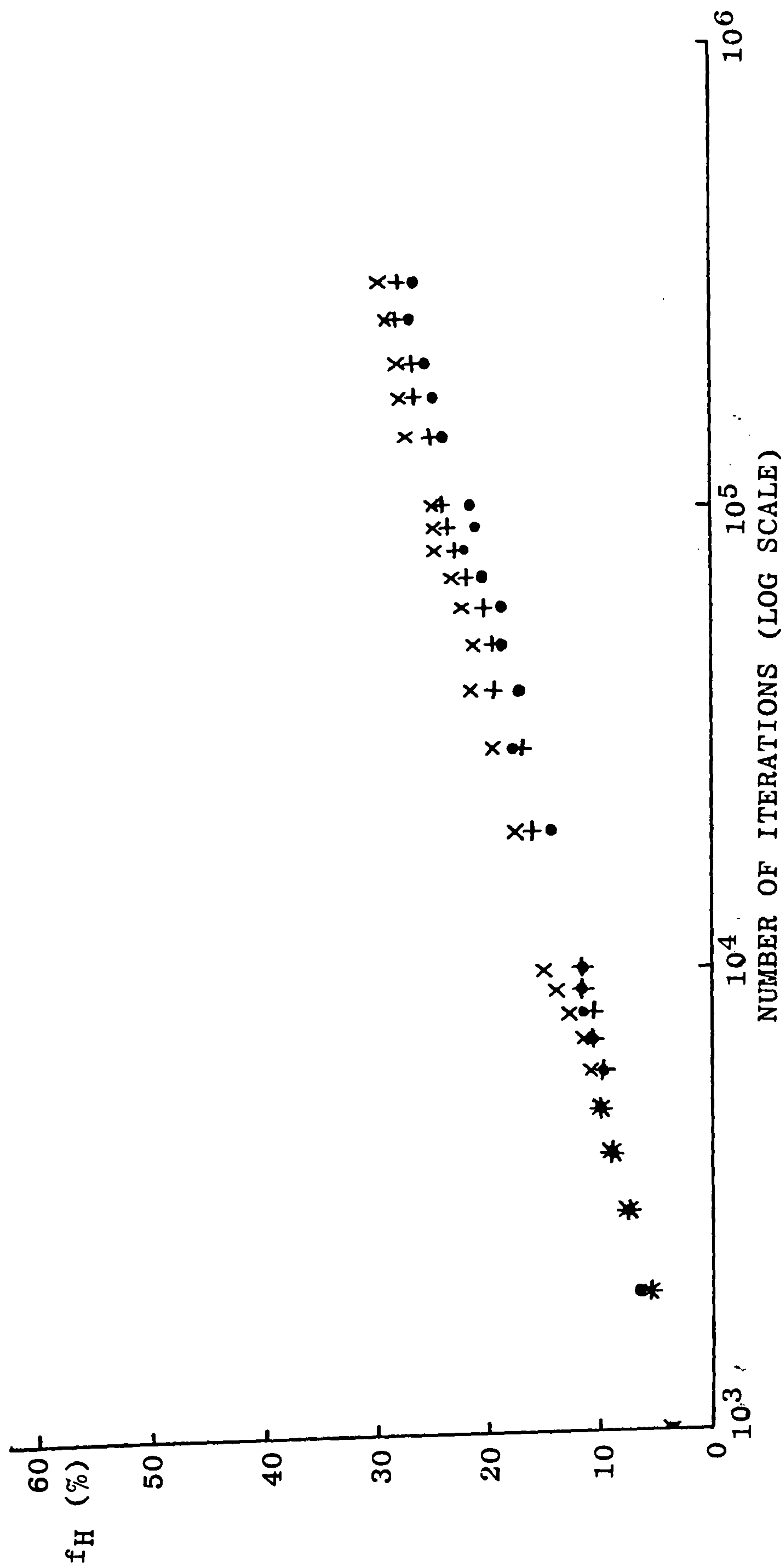


Figure 7.8. The effect of varying  $p(\text{inter})$  on the shapes of the simulated renaturation curves at a quench temperature of  $20^{\circ}\text{C}$ . Values of  $p(\text{inter})$  illustrated are 0.1 (●), 1 (+) and 10 (x). Other program parameters are:  
 $x_c = 1$ ,  $T_c = 36^{\circ}\text{C}$ , no. of cycles = 300,000, no. of chains = 200,  $\text{PKINK} = 0.15$ ,  $\text{PTRANS} = 0$ ,  $\Delta H_{\text{cal}} = 12.8 \text{ kJ mol}^{-1}$ .

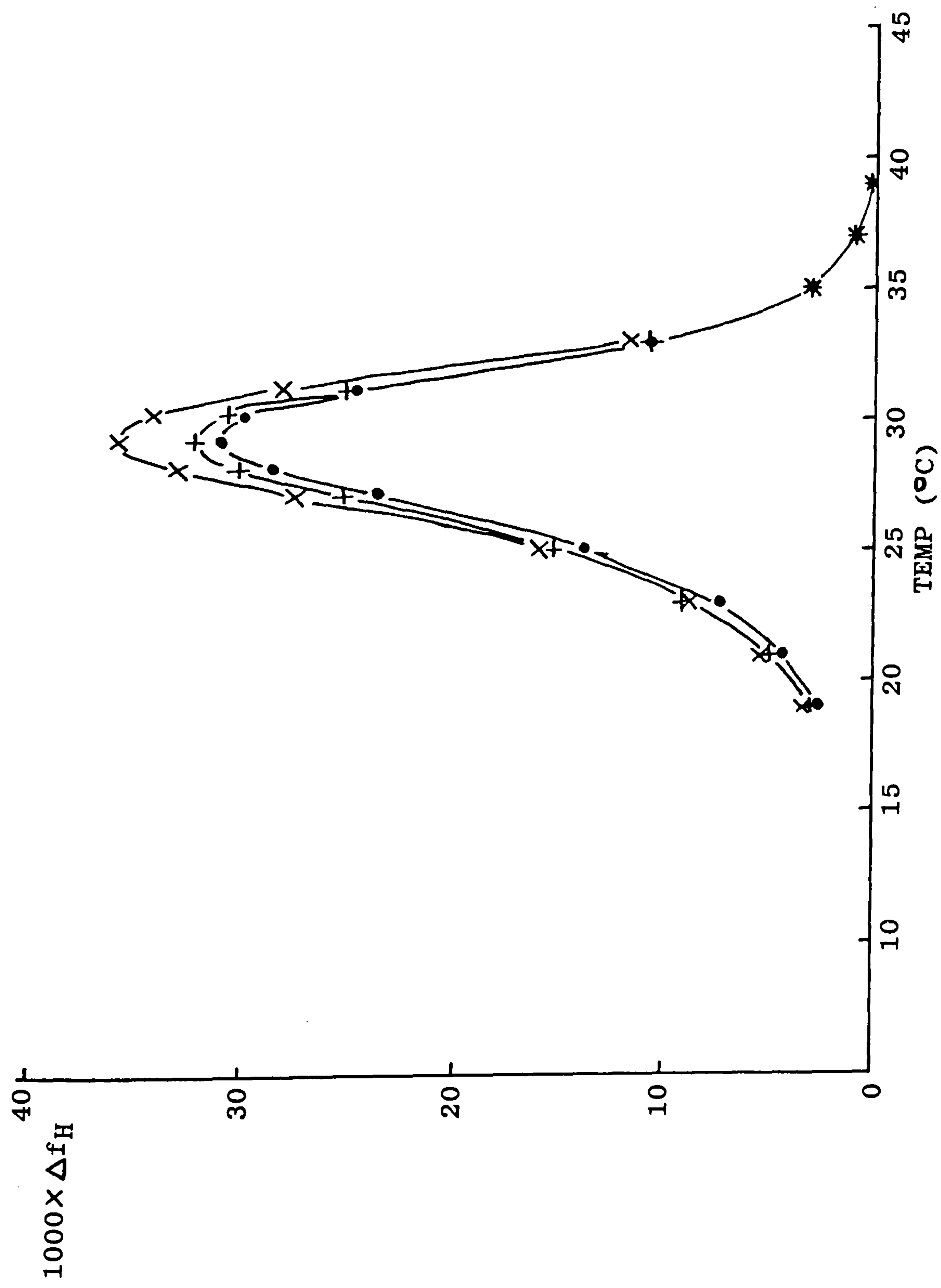


Figure 7.9. The effect of varying  $p(\text{inter})$  on the shape of simulated melting profiles. The melting profiles shown correspond to the simulated renaturations in Fig. 7.8.

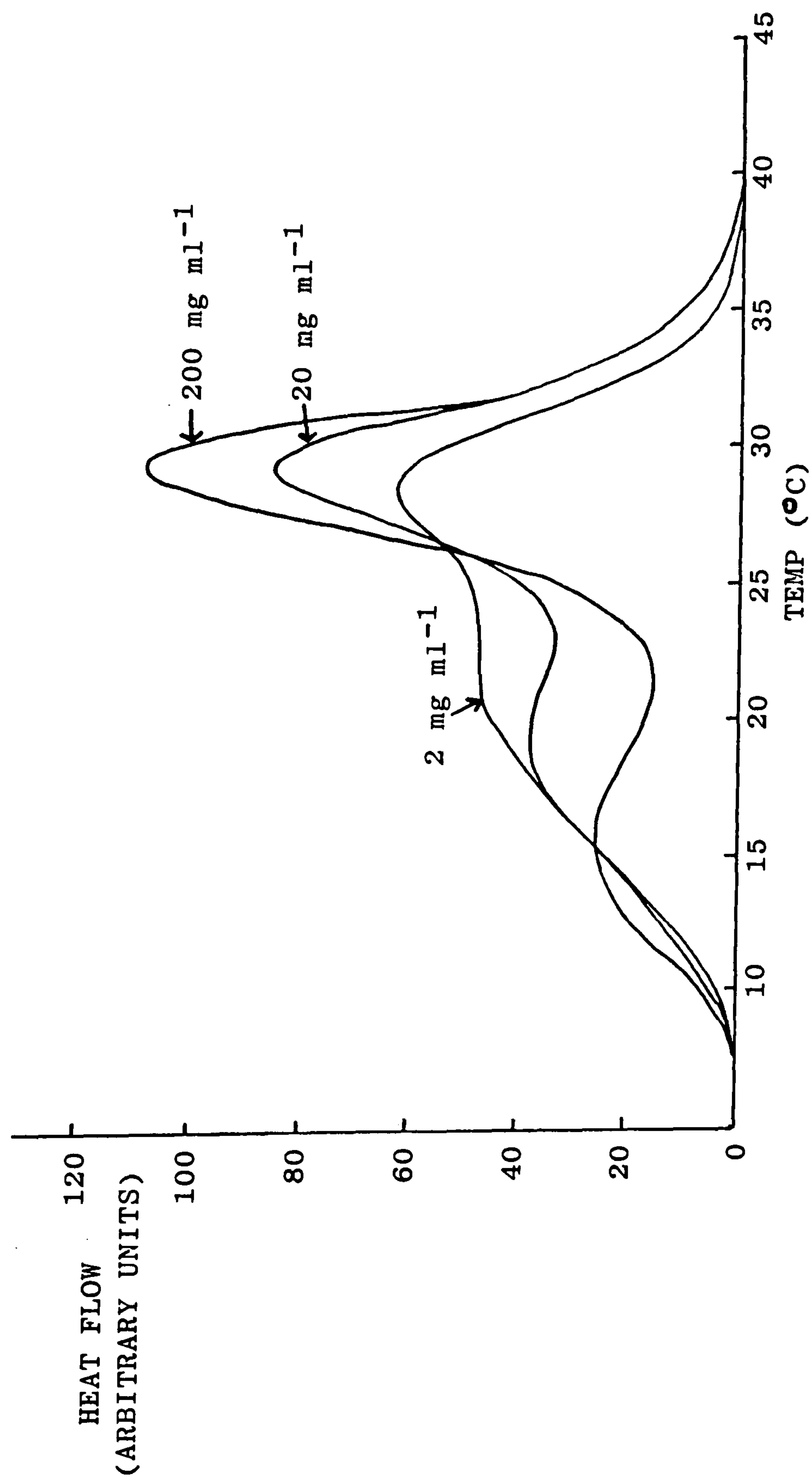


Figure 7.10. DSC melting profiles of gelatin at different concentrations (2, 20 and 200 mg ml<sup>-1</sup>) after cooling to 20°C for 24 hours followed by a further 16 hours at 5°C. The plots are superimposed onto the same arbitrary scale and illustrate the increase in size of the higher melting peak with increase in gelatin concentration.



outcome of simulation attempts. In the next section, the four remaining variables, having the most significant effect on the results from the simulation, are considered.

#### 7.6.2. Dominant Parameters

In Section 7.3, during the discussion of the helix stability test, a parameter  $x$  was defined representing the number of units (i.e. three amino acid residues, one from each chain) incapable of participation in bonding as a result of end effects. Values of  $x$  (varying between 1 and 4) have been used to observe the effect of the parameter on simulated helix fractions and melting profiles. Qualitatively, increasing  $x$  decreases helix stability (by reducing the proportion of residues contributing to the bonding scheme), especially in shorter helices where end effects become particularly significant. The length distribution of helices generated in the simulation can be increased by increasing the parameter PTRANS (which defines the probability of helix propagation beyond 'cis kinks'), giving a means of offsetting the destabilising effect of high values of  $x$ . The interplay between these two parameters is explored in Figs. 7.11 and 7.12. Fig. 7.11a illustrates the effect of an increase in PTRANS on the simulated melting point for various values of  $x$ . In this plot the simulation was carried out over 300,000

iterations and at a quench temperature of 5°C. The 'target value' of the melting point obtained from optical rotation measurements is also shown on the plot. The graph illustrates that, as expected, an increase in PTRANS causes the melting point to increase and also that an increase in  $x$  causes a reduction in the simulated melting point. Consequently, various combinations of  $x$  and PTRANS are capable of producing simulated melting points with values the same as those obtained experimentally. However, the helix fractions generated after a large number of iterations, under conditions producing the best simulated melting points, are considerably larger than the experimental helix fractions after long periods of time at the quench temperature. In fact, in contrast to the melting points, only small values of PTRANS combined with high values of  $x$  are capable of producing the experimentally-observed helix fractions (Fig. 7.11b). Similarly, at quench temperatures of 25°C, the target values for melting points are best achieved using low values of  $x$  (Fig. 7.12a), while the helix fraction target values require high values of  $x$  (Fig. 7.12b).

The simulated helix fractions are also dependent on the value of PKINK and the melting point of the parent collagen ( $T_c$ ) and, using these two parameters, it is possible to match the experimental and simulated helix fractions for any reasonable value of  $x$ . However, lower values of  $x$  appeared to be required to match simulated



melting points with experimental values for the same quench temperatures.

A further indication favouring lower values of  $x$  came from the co-operativity of the simulated melting transition which, as shown in Fig. 7.13, became considerably sharper for larger values of  $x$ . In real gelatin systems, the transition is not so sharp (see Chapter 5), and consequently large values of  $x$  appeared unlikely, with a value of  $x = 1$  producing melting profiles most similar in shape to those obtained experimentally.

Theoretically, a value of  $x = 1$  would also be reasonable when considering the hydrogen bonded structures proposed for the collagen triple helix (see Fig. 2.5). In these structures there is one hydrogen bond per unit, except for the last unit in the helix which is incapable of participation in hydrogen bonding (thereby indicating a value of  $x = 1$ ).

A value of  $x = 1$  was therefore adopted, leaving three parameters, PTRANS, PKINK and  $T_c$  as the only significant variables. As discussed in Section 7.5, however, PKINK and PTRANS can be regarded, for simulation purposes, as behaving mathematically in the same manner. For this reason PTRANS was set to zero and PKINK used as the only variable simulating the effect of 'cis kinks'.



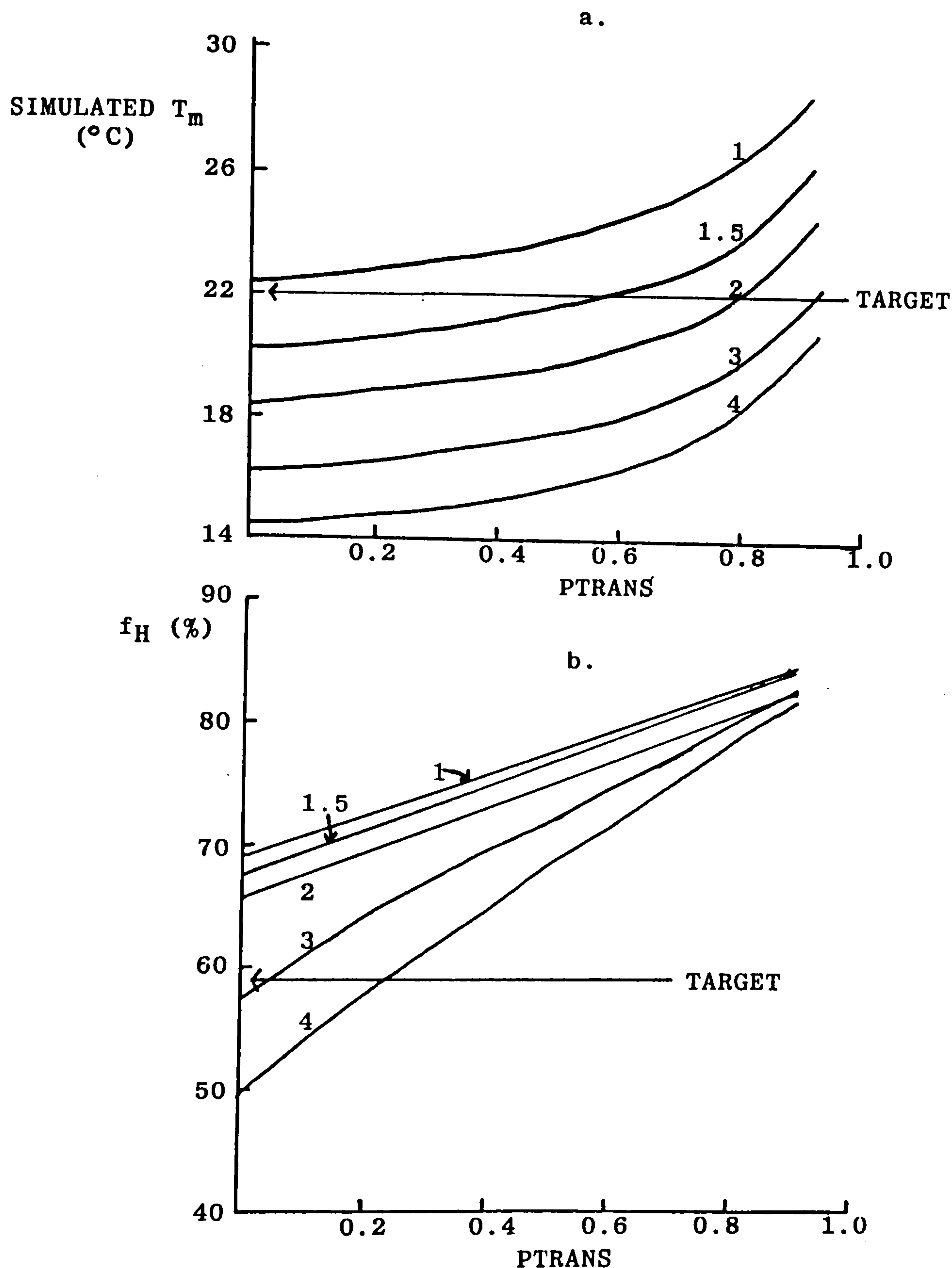


Figure 7.11. a) The variation in simulated melting point obtained as a function of PTRANS for varying values of  $x$  at a renaturation temperature of 5°C. b) The variation in simulated helix fraction (after a large number of iterations) as a function of PTRANS for varying values of  $x$  at a renaturation temperature of 5°C.

Values of  $x$  are indicated on the plots along with the 'target values' from optical rotation measurements (arrows). Other program parameters are  $T_c = 36^\circ\text{C}$ , no. of cycles = 300,000, no. of chains = 100,  $p(\text{inter}) = 1$ ,  $\text{PKINK} = 0.1$ ,  $\Delta H_{ca} = 12.8 \text{ kJ mol}^{-1}$ .

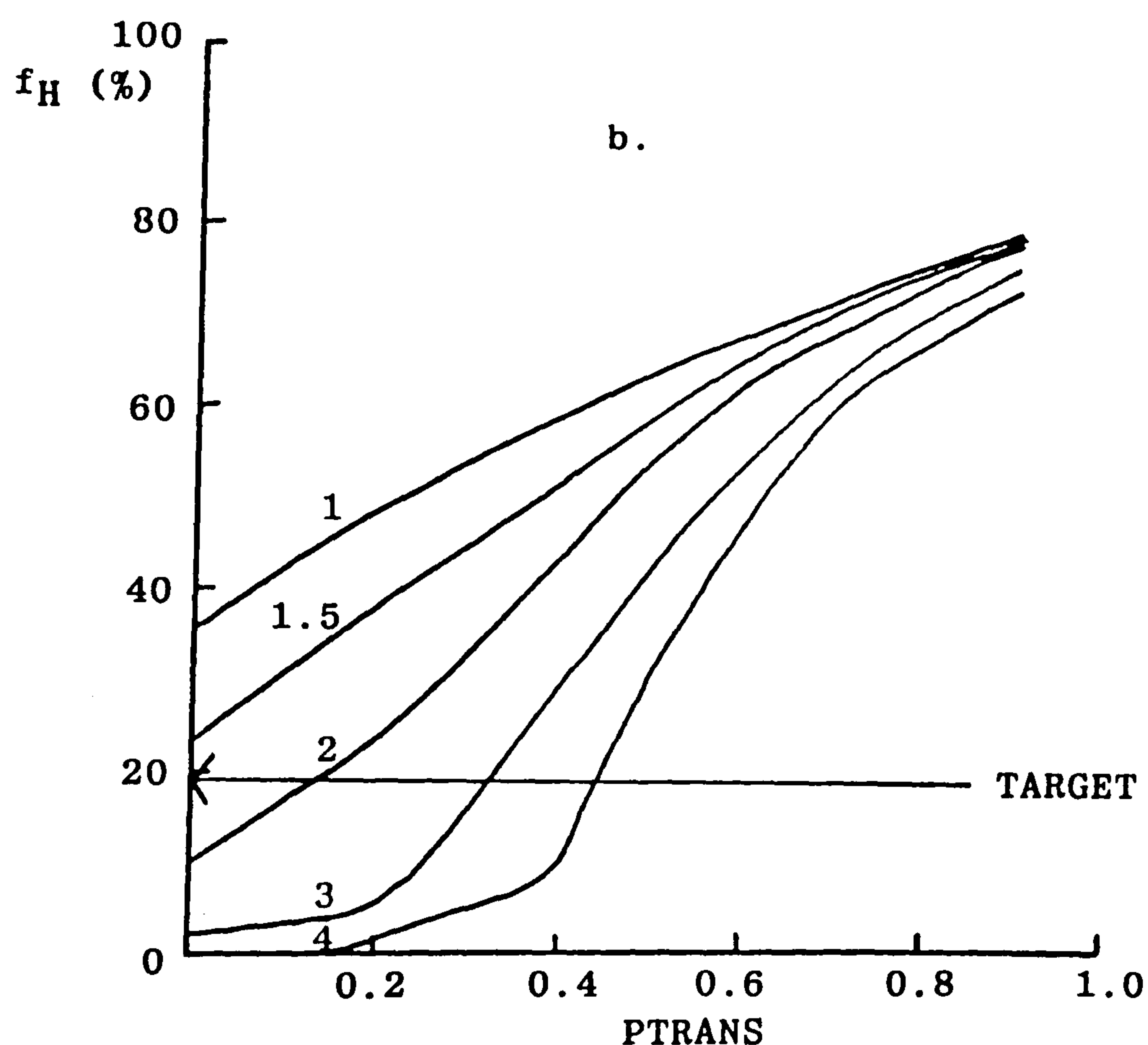
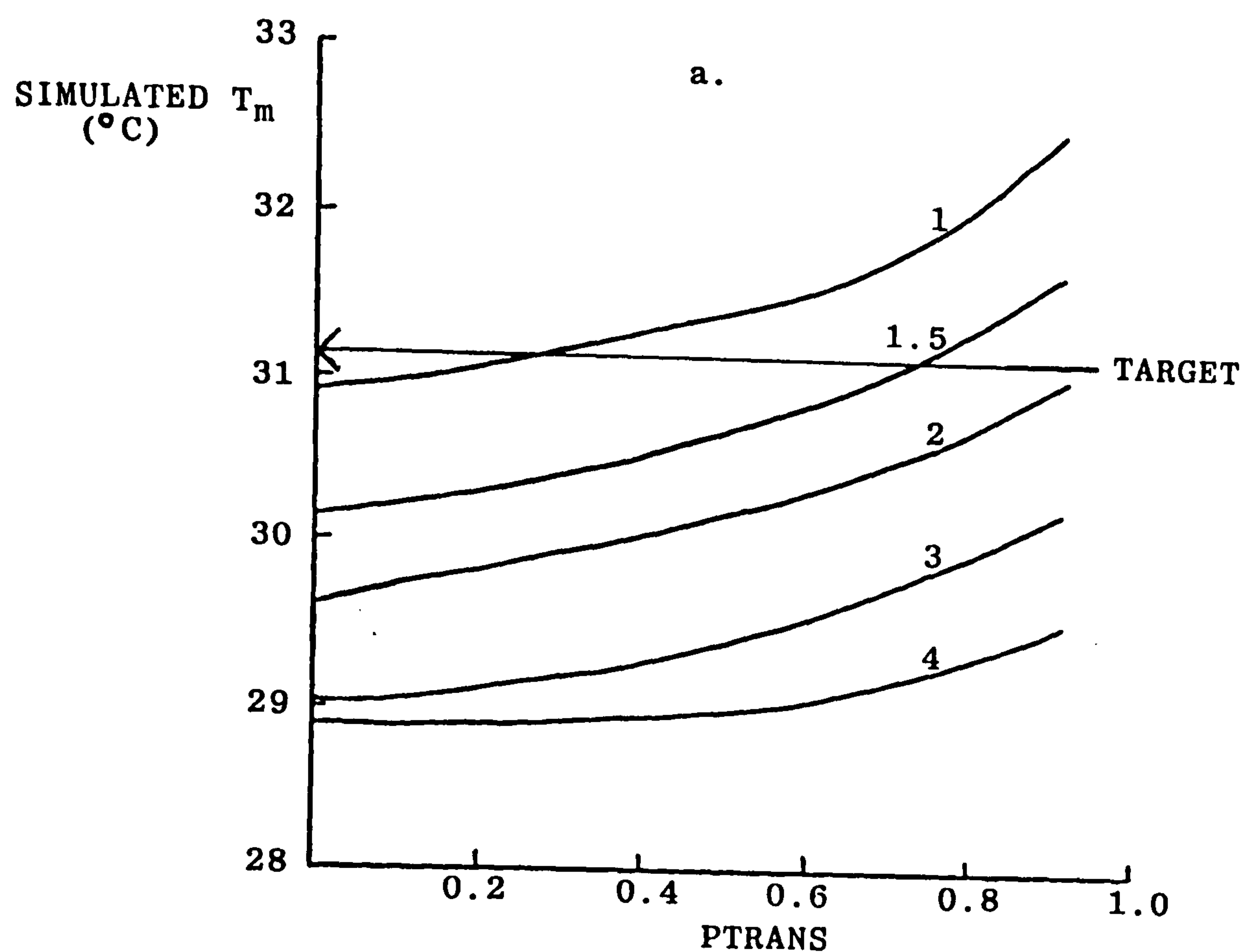


Figure 7.12. The variation in simulated melting point (a) and simulated helix fraction (b) as a function of PTRANS for varying values of  $x$  at a renaturation temperature of 25°C. Other program parameters are as in Fig. 7.11. Values of  $x$  are indicated on the plots along with the 'target values' from optical rotation measurements (arrows).

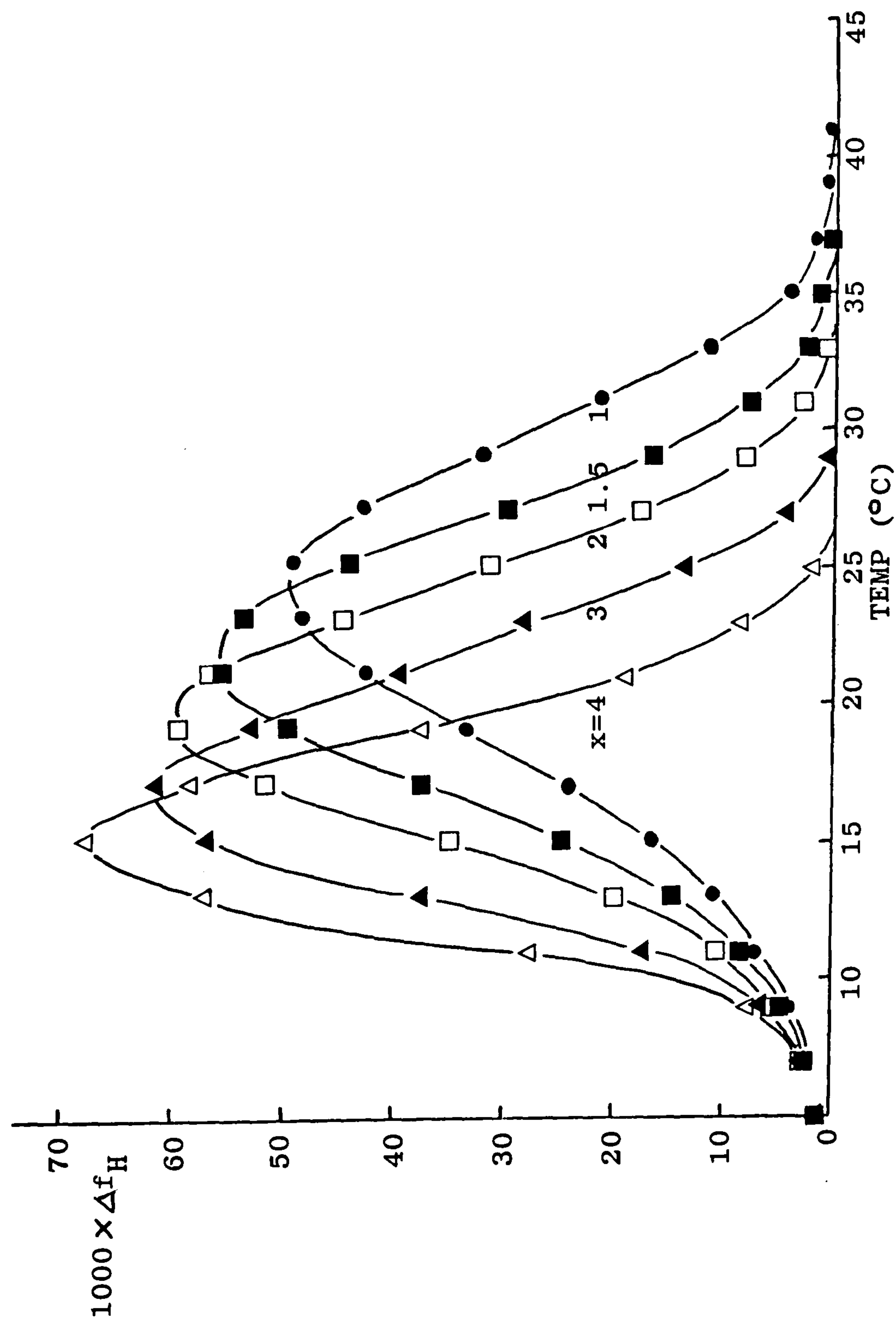


Figure 7.13. Effect of varying the value of  $x$  on the shape of the simulated melting transition at  $5^{\circ}\text{C}$ . With increasing  $x$  the width of the melting peak decreases as is illustrated for values of  $x$  of 1, 1.5, 2, 3 and 4. Other program parameters are  $T_c = 36^{\circ}\text{C}$ ,  $\text{PKINK} = 0.1$ ,  $\text{PTRANS} = 0.2$ , no. of chains = 100, no. of iterations = 300,000,  $p(\text{inter}) = 1$ ,  $\Delta H_{\text{cal}} = 12.8 \text{ kJ mol}^{-1}$ .



The effect of PKINK on simulated helix fractions and melting temperatures at various quench temperatures is illustrated in Figs. 7.14, 7.15, 7.16 and 7.17 for  $T_c$  values of 36, 37, 38 and 39°C, respectively. The values of the other program parameters are indicated on the plots. The plots all show the same general trend, with values of PKINK in the region of 0.10 to 0.17 giving helix fractions and melting temperatures similar to those observed experimentally. For  $T_c = 36^\circ\text{C}$  (Fig. 7.14), the values of PKINK necessary to produce the target helix fractions and melting points span quite a broad range, from 0.08 for the target melting point at 25°C to 0.17 for the target helix fraction at 5°C. However, with increasing  $T_c$ , the span of PKINK required to produce the target values for all quench temperatures and helix fractions decreases (as shown in Figs. 7.15 and 7.16 for  $T_c = 37$  and 38°C, respectively) before beginning to increase again (at  $T_c = 39^\circ\text{C}$ ; Fig. 7.17) due to the high values of PKINK required to achieve the target melting temperatures.

The combination of  $T_c$  and PKINK that produces the best simulations of both experimental helix fractions and melting temperatures therefore consists of a  $T_c$  between 37°C and 38°C and a value of PKINK somewhere in the range 0.14 - 0.17. This value of PKINK can be regarded as the lower limit for the *trans-cis* isomerisation equilibrium constant as a result of PTRANS

being set to zero. If, however, in a real system the probability of overcoming a 'cis kink' is not zero then the true value of the *trans-cis* equilibrium constant would be somewhat higher.

The simulated helix regeneration curves and melting curves for quench temperatures of 5, 15, 20 and 25°C, using a value for PKINK of 0.15 and a melting point for collagen of 38°C are presented in Figs. 7.18a and b, respectively. Also shown are the experimental helix regeneration curves (Fig. 7.19a) and melting curves (Fig. 7.19b) for the same quench temperatures, obtained from optical rotation measurements. There is clearly excellent agreement between the simulated and experimental results. Not only are the maxima of the melting profiles at approximately the same temperature, but also, the sizes and shapes of the peaks correspond closely. In the plots of simulated helix regeneration, where the number of iterations is considered as representing time, there is also agreement in general shape and features between the experimental and simulated results. The discrepancies between the two generally occur at short times (or low numbers of iterations) and can easily be explained.

In the real system, nucleation and helix growth can occur simultaneously in all of the chains, as soon as the quench temperature is reached, thereby giving rise to much larger helix fractions in the initial stages



than can be simulated in the Monte Carlo program, which is capable of ordering only one chain at a time. In the later stages of renaturation, however, where the annealing-in of structures has a greater effect, there is extremely good agreement between the simulated and experimental renaturation curves. This is particularly encouraging, since in previous studies the long-time aging process has been the most difficult part of the renaturation curve to simulate.

Finally, not only has the simulation program been capable of reproducing the helix fractions and melting curves observed experimentally for samples quenched from the sol state to specific temperatures, but also it has been able to reproduce the melting behaviour of gels formed by holding for a period of time at some intermediate temperature prior to cooling to the final quench temperature (i.e. the biphasic melting behaviour discussed in Chapter 5).

This is illustrated in Fig. 7.20 for simulated renaturations involving 300,000 and 80,000 iterations at 25°C followed by a further 300,000 iterations at 5°C. The values of all the other program parameters are indicated on the plot. The melting profiles are very similar to those observed experimentally (Fig. 7.21) for gels cured at 25°C for varying lengths of time followed by 16 hours at 5°C, further emphasizing the quality of results attainable from the simulation program.



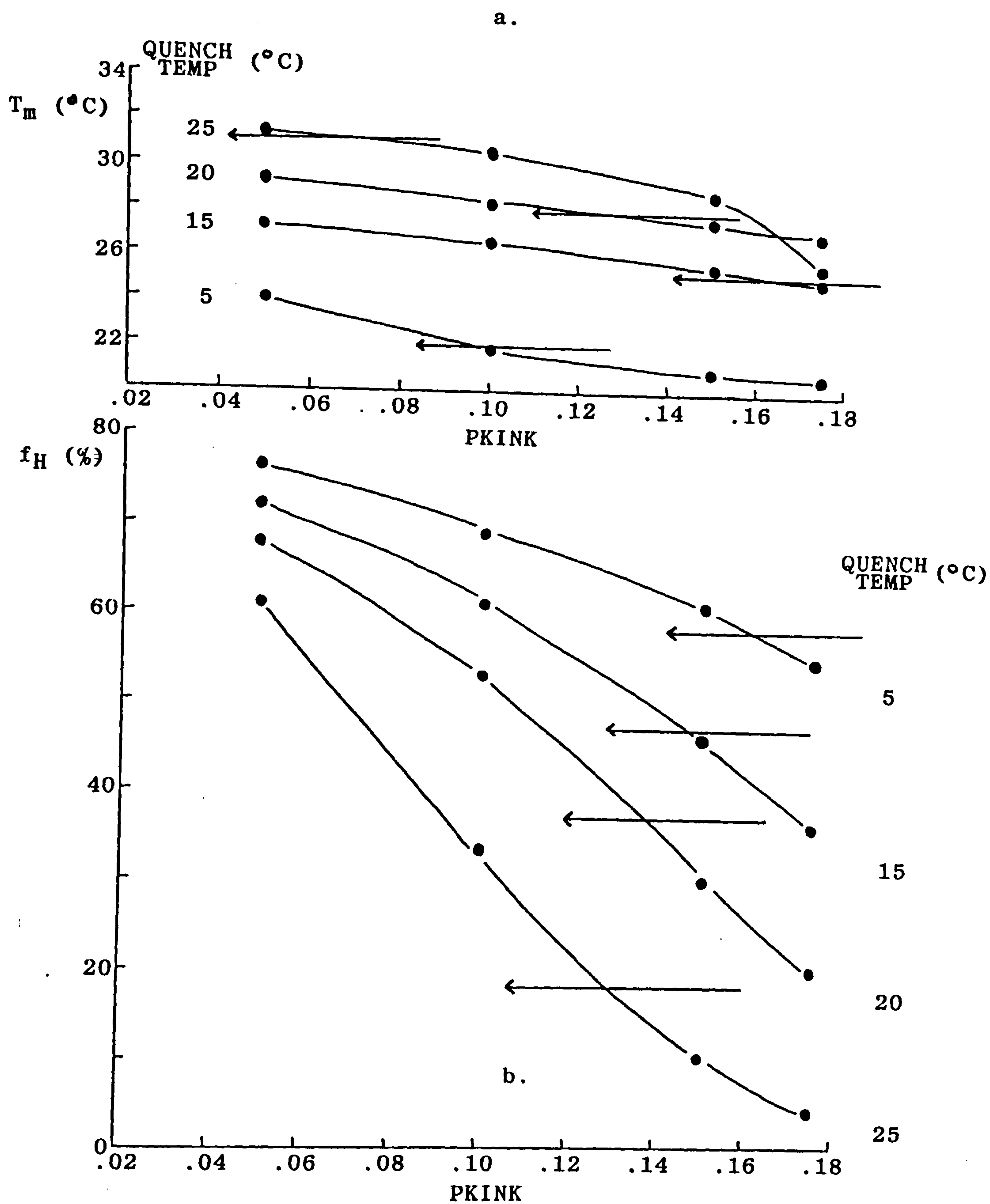


Figure 7.14. The effect of varying PKINK on the melting temperatures (a) and the helix fractions (b) for various quench temperatures (5, 15, 20, 25°C) in simulations using a value of 36°C for  $T_c$ . The 'target values' for the melting temperatures and helix fractions from optical rotation are indicated for each quench temperature (by arrows). Other program parameters are  $x = 1$ , PTRANS = 0, p(inter) = 10, no. of iterations = 300,000, no. of chains = 200.

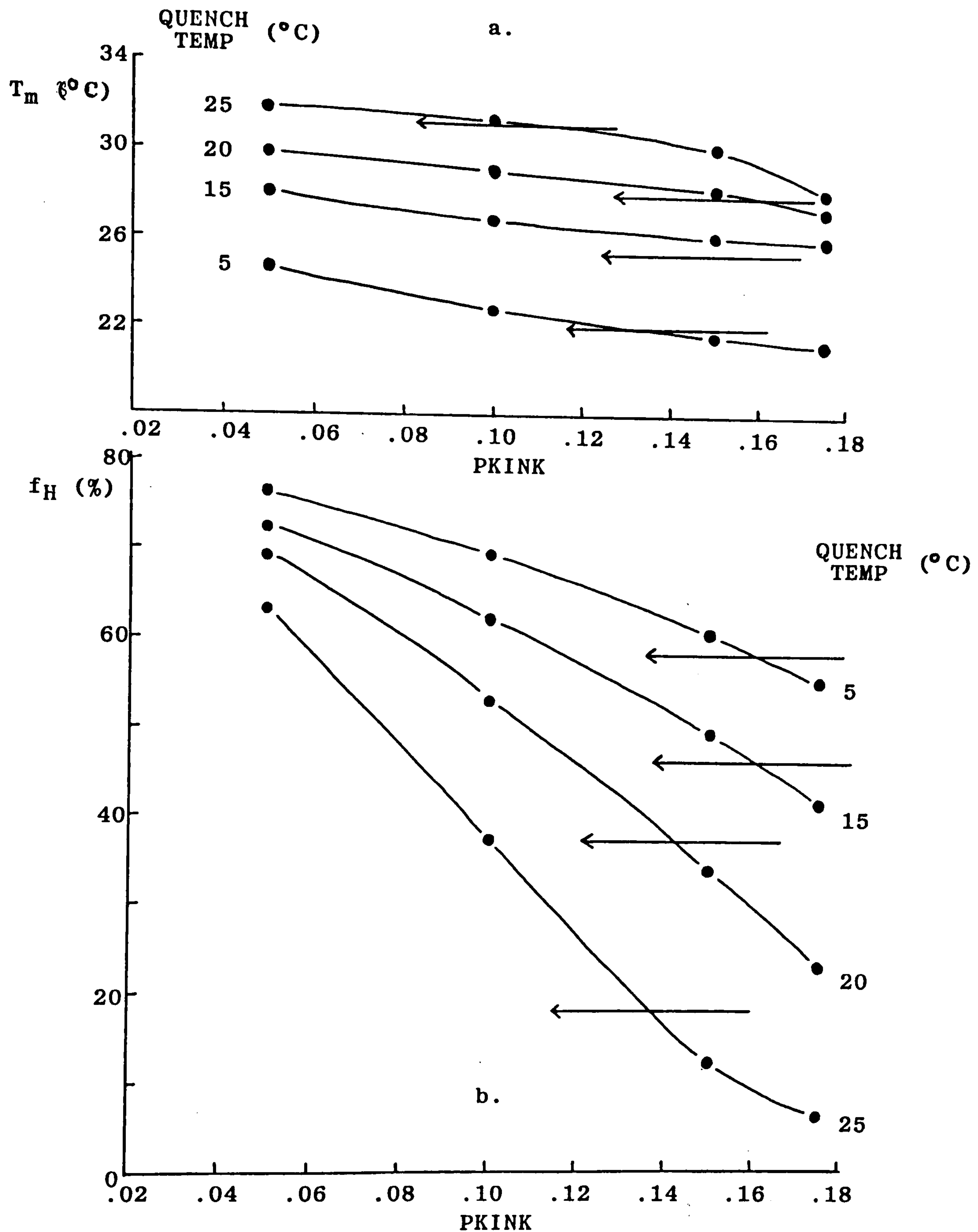


Figure 7.15. The effect of varying PKINK on the melting temperatures (a) and the helix fractions (b) for various quench temperatures (5, 15, 20, 25  $^{\circ}\text{C}$ ) in simulations using a value of 37  $^{\circ}\text{C}$  for  $T_c$ . The 'target values' for the melting temperatures and helix fractions from optical rotation are indicated for each quench temperature (by arrows). Other program parameters are  $x = 1$ , PTRANS = 0, p(inter) = 10, no. of iterations = 300,000, no. of chains = 200.

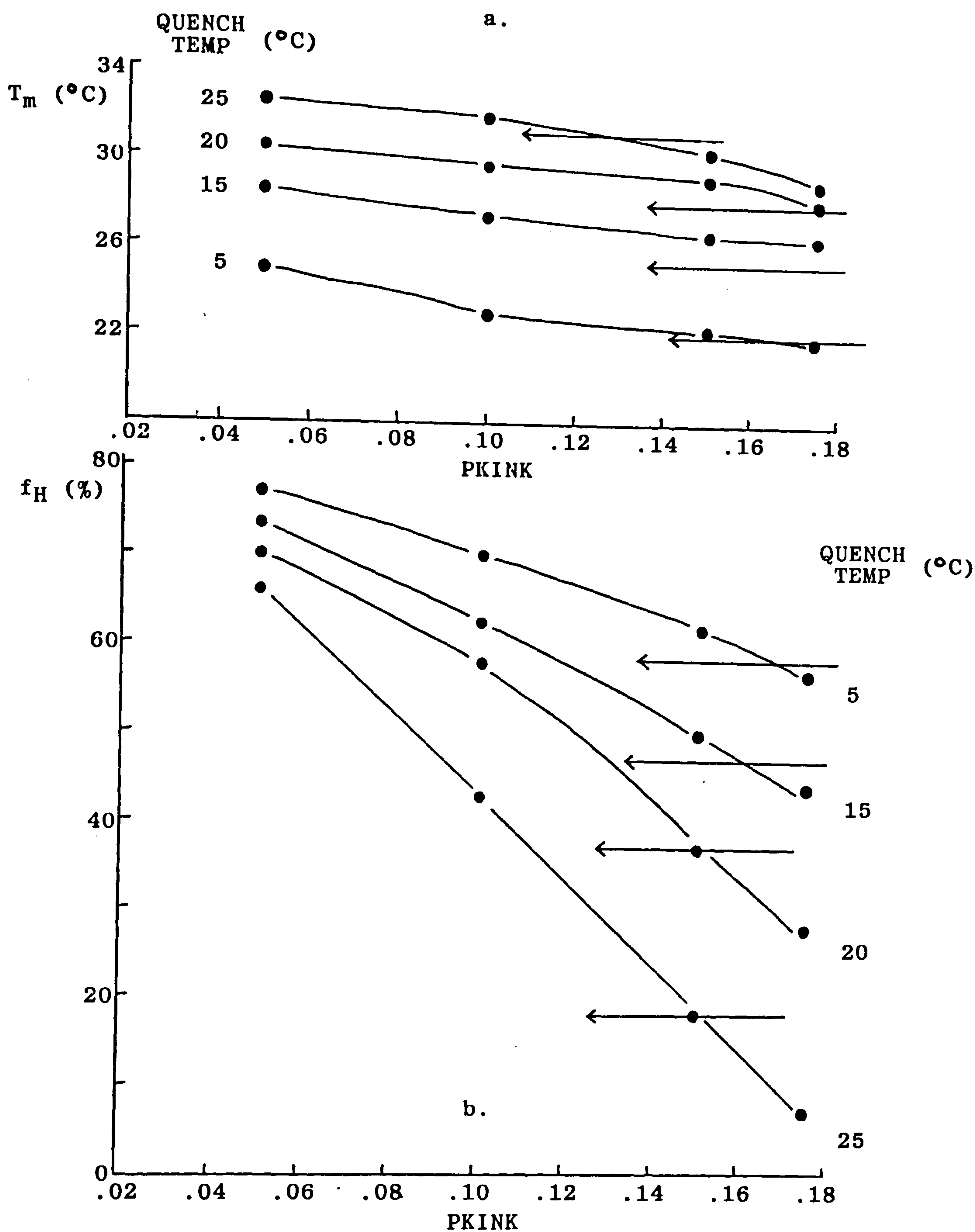


Figure 7.16. The effect of varying PKINK on the melting temperatures (a) and the helix fractions (b) for various quench temperatures (5, 15, 20, 25  $^{\circ}\text{C}$ ) in simulations using a value of 38  $^{\circ}\text{C}$  for  $T_c$ . The 'target values' for the melting temperatures and helix fractions from optical rotation are indicated for each quench temperature (by arrows). Other program parameters are  $x = 1$ , PTRANS = 0,  $p(\text{inter}) = 10$ , no. of iterations = 300,000, no. of chains = 200.



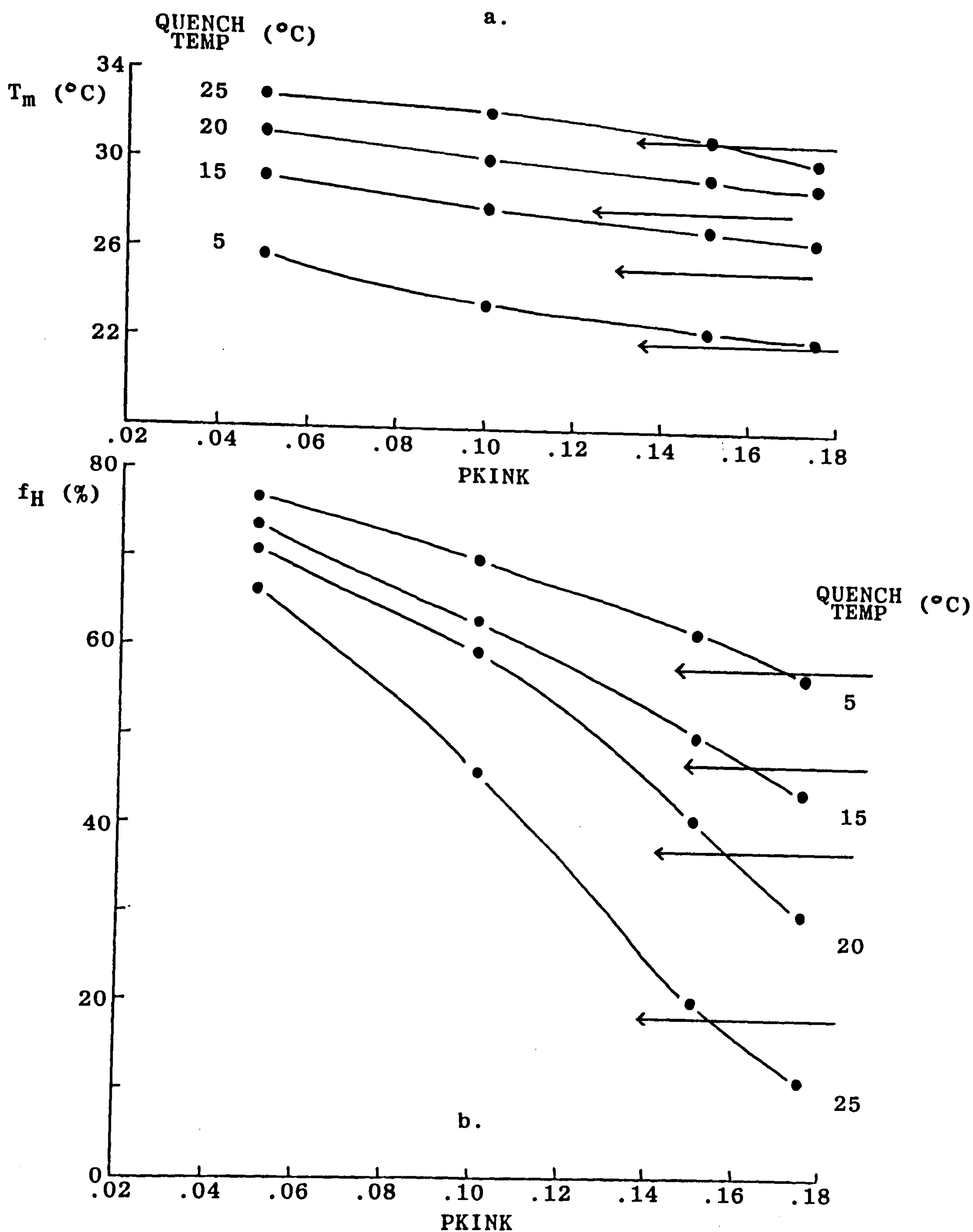


Figure 7.17. The effect of varying PKINK on the melting temperatures (a) and the helix fractions (b) for various quench temperatures (5, 15, 20, 25°C) in simulations using a value of 39°C for  $T_c$ . The 'target values' for the melting temperatures and helix fractions from optical rotation are indicated for each quench temperature (by arrows). Other program parameters are  $x = 1$ , PTRANS = 0,  $p(\text{inter}) = 10$ , no. of iterations = 300,000, no. of chains = 200.

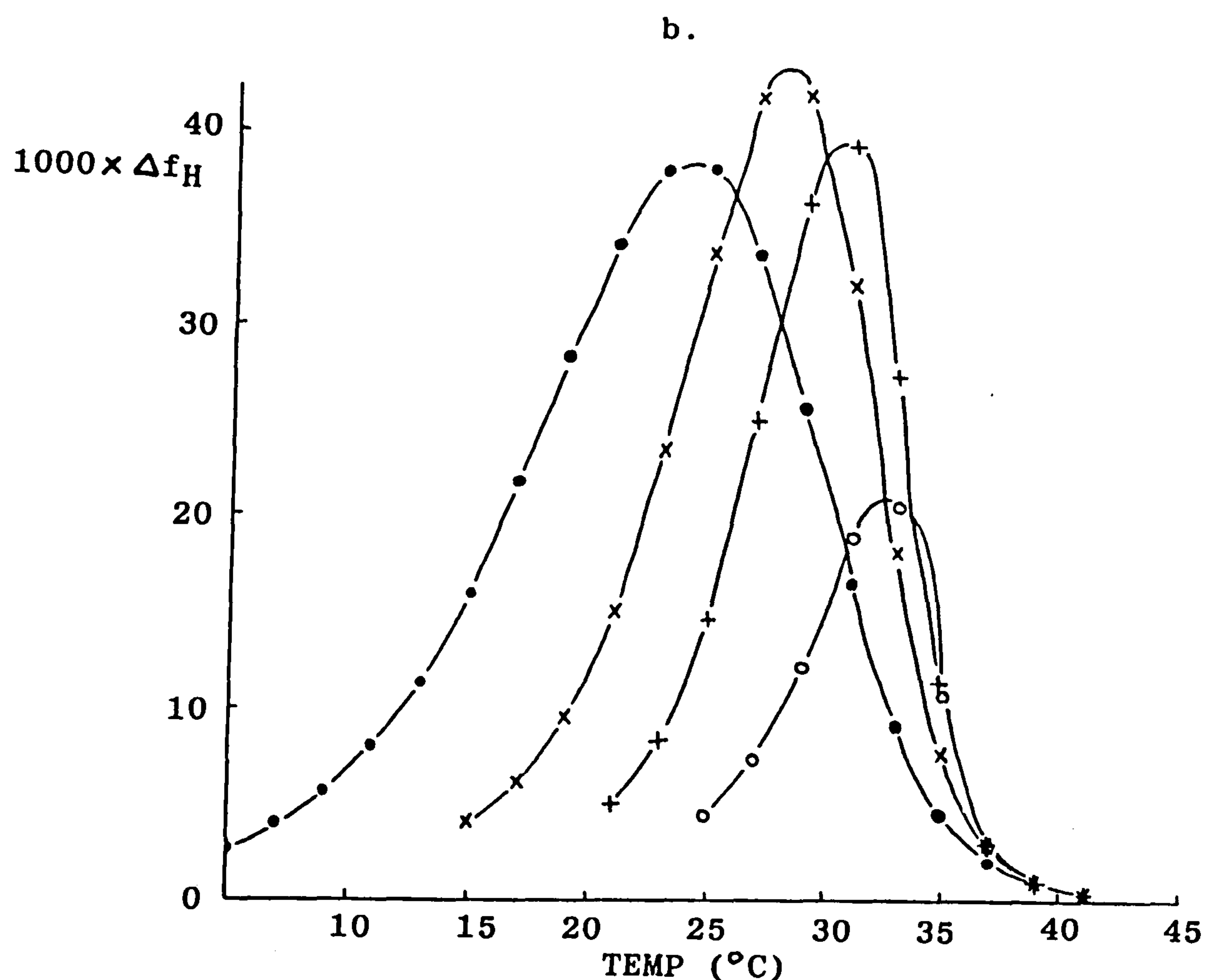
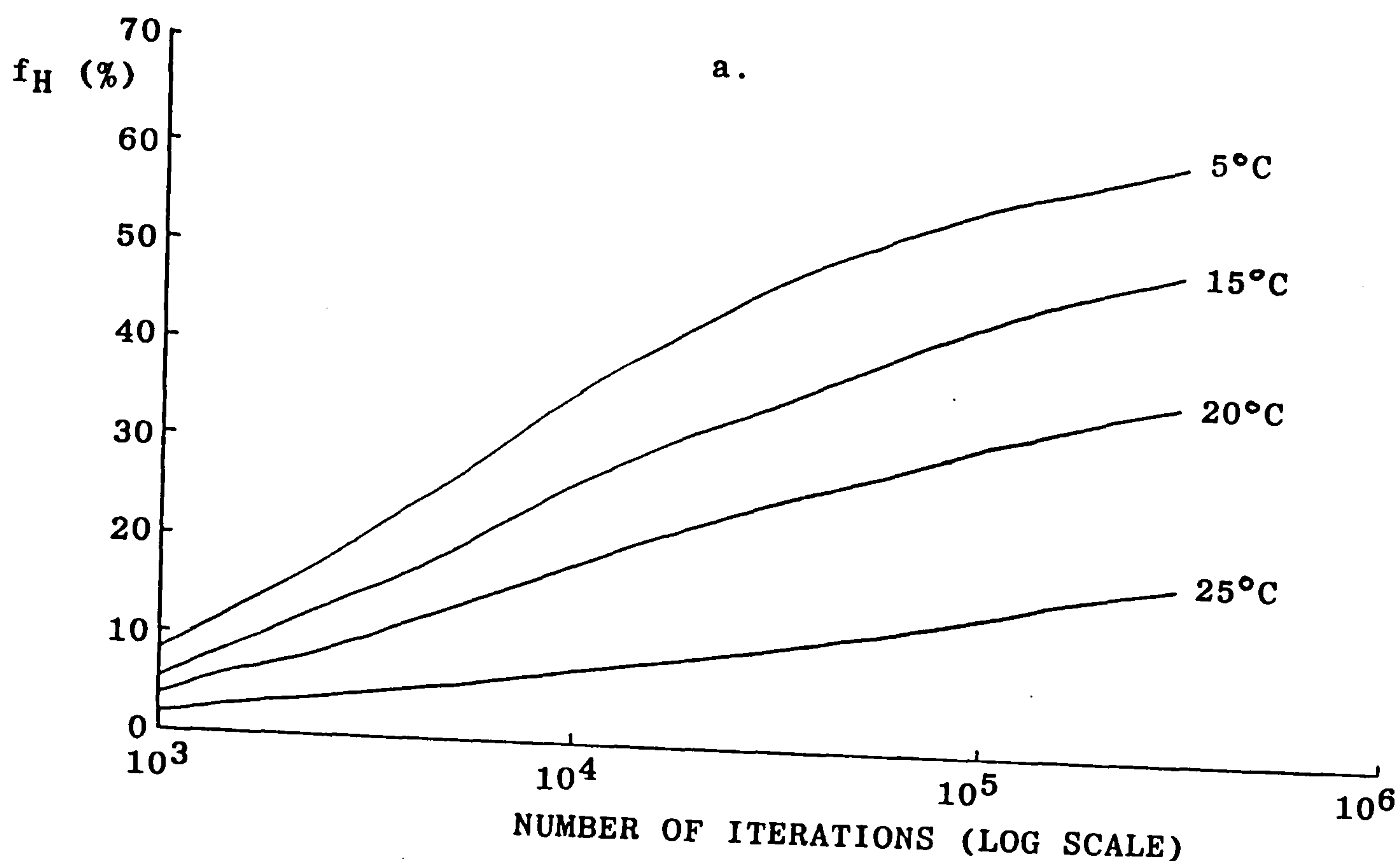


Figure 7.18. a) Simulated helix regeneration curves for quench temperatures of 5, 15, 20 and 25°C. b) Simulated melting curves for quench temperatures of 5 (●), 15 (×), 20 (+) and 25 (○)°C after a large number of iterations. Plots obtained using the best combination of program parameters (i.e.  $x = 1$ ,  $PKINK = 0.15$ ,  $PTRANS = 0$ ,  $T_c = 38^\circ\text{C}$ ,  $p(\text{inter}) = 10$ , no. of iterations = 300,000, no. of chains = 200).

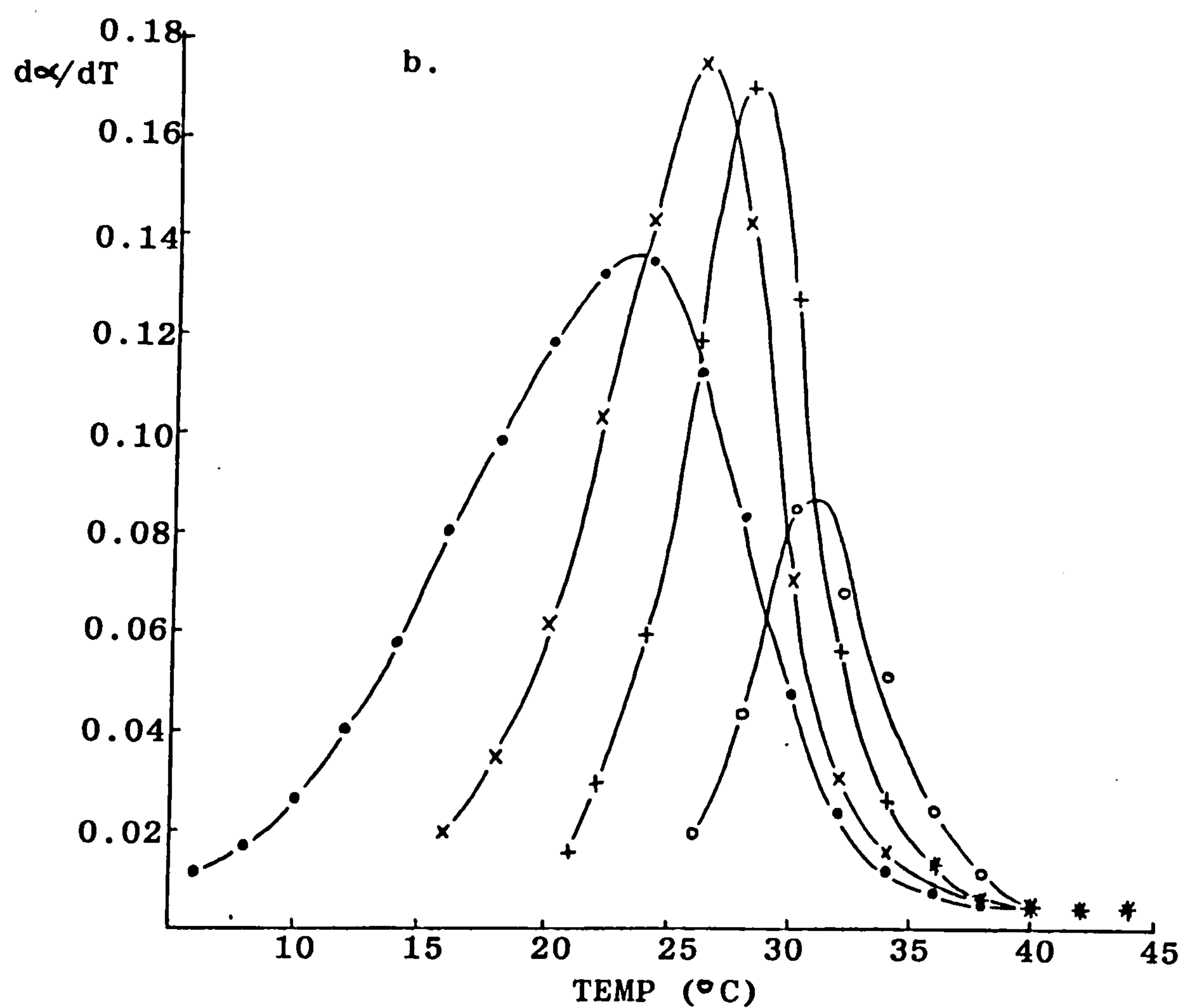
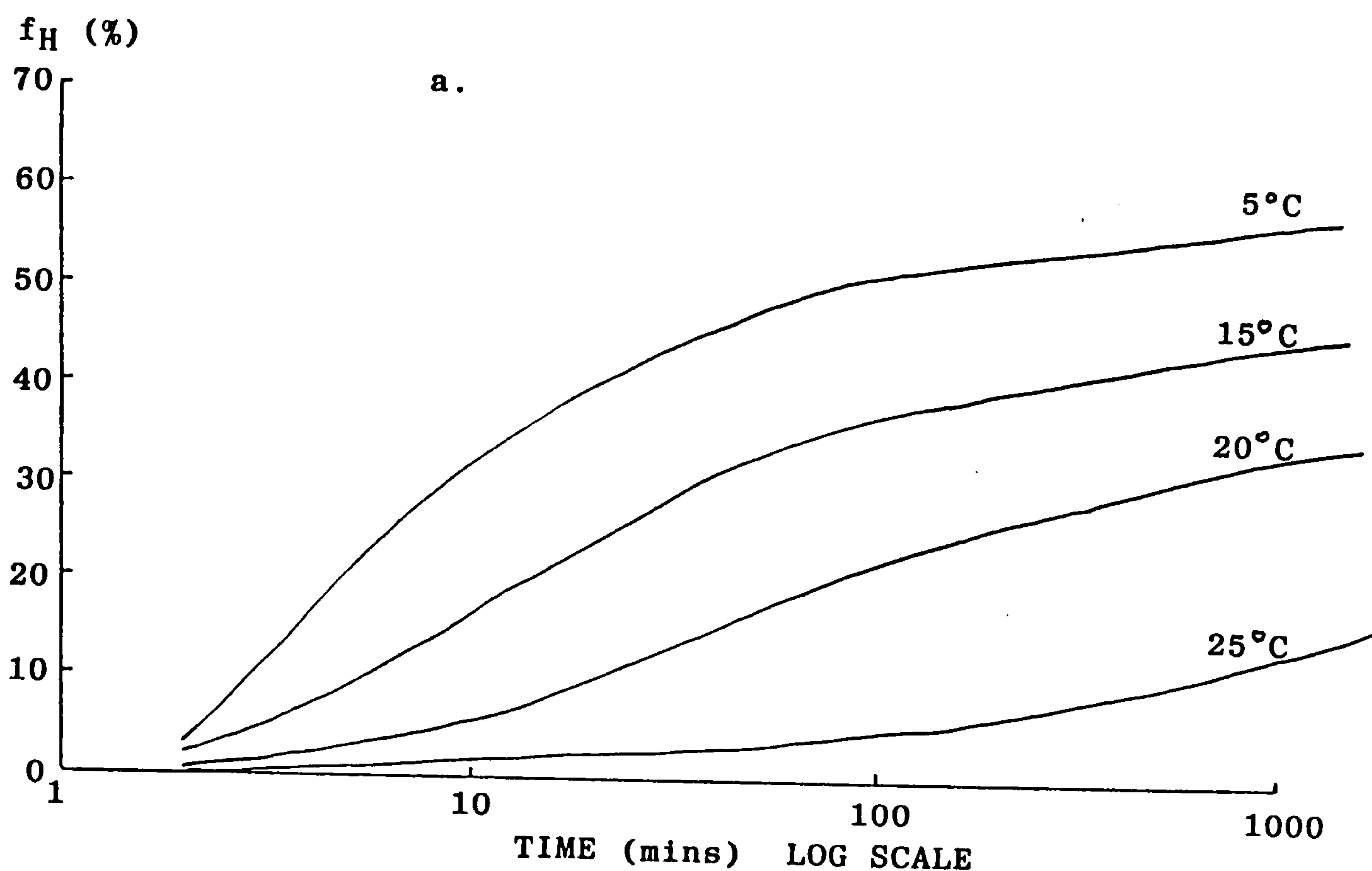


Figure 7.19. a) Regeneration of helix fraction (from optical rotation) as a function of time on quenching an LO-1 solution ( $20 \text{ mg ml}^{-1}$ ) to temperatures of 5, 15, 20, 25°C. b) Melting profiles of LO-1 gels ( $20 \text{ mg ml}^{-1}$ ) as monitored by optical rotation, for gels formed at 5 (●), 15 (X), 20 (+) and 25 (○)°C.



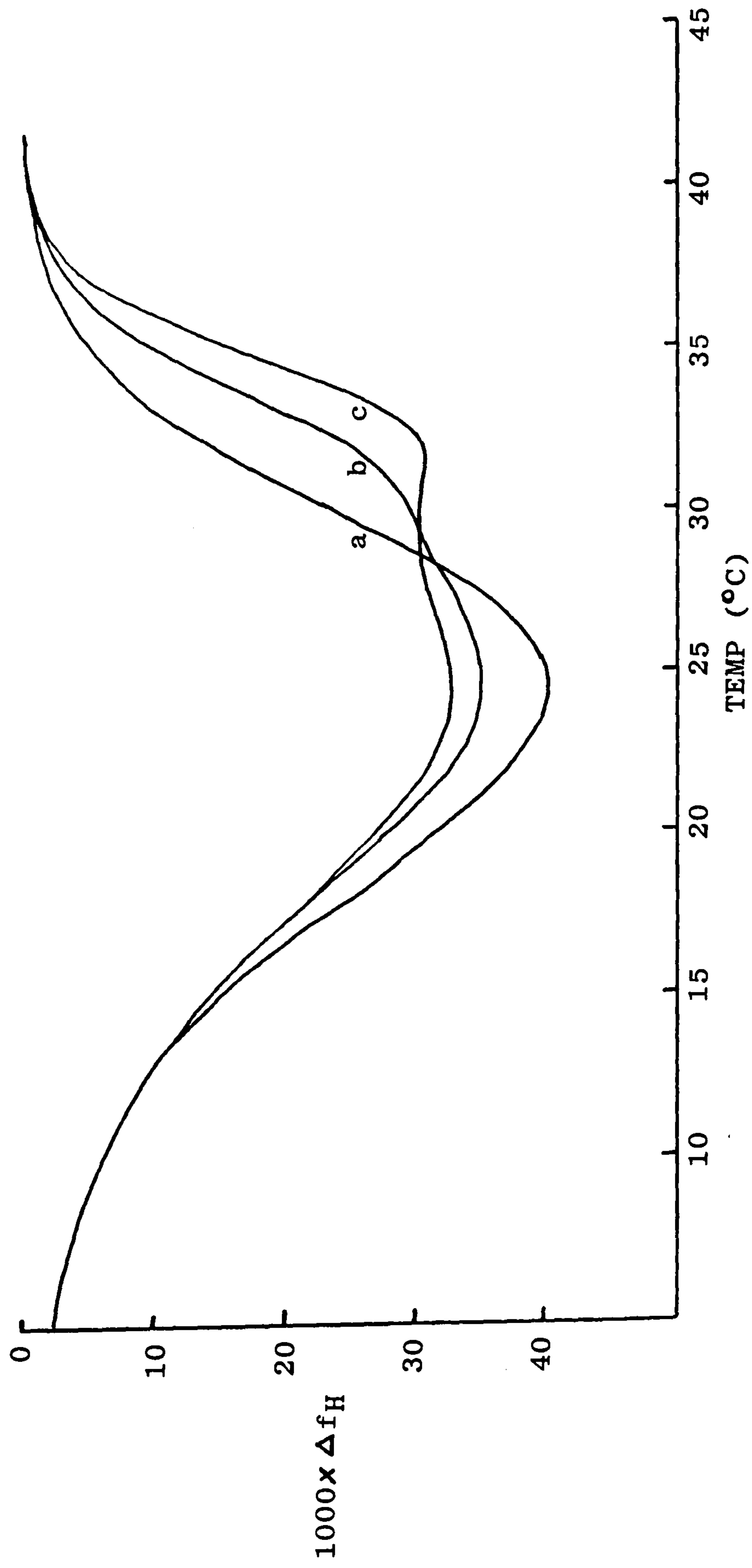


Figure 7.20. Simulated melting profiles obtained from the simulation program after varying numbers of iterations at a quench temperature of 25 °C followed by 300,000 iterations at a temperature of 5°C: a) zero iterations at 25°C; b) 80,000 iterations at 25°C; c) 300,000 iterations at 25°C. Other program parameters are as in Fig. 7.18.

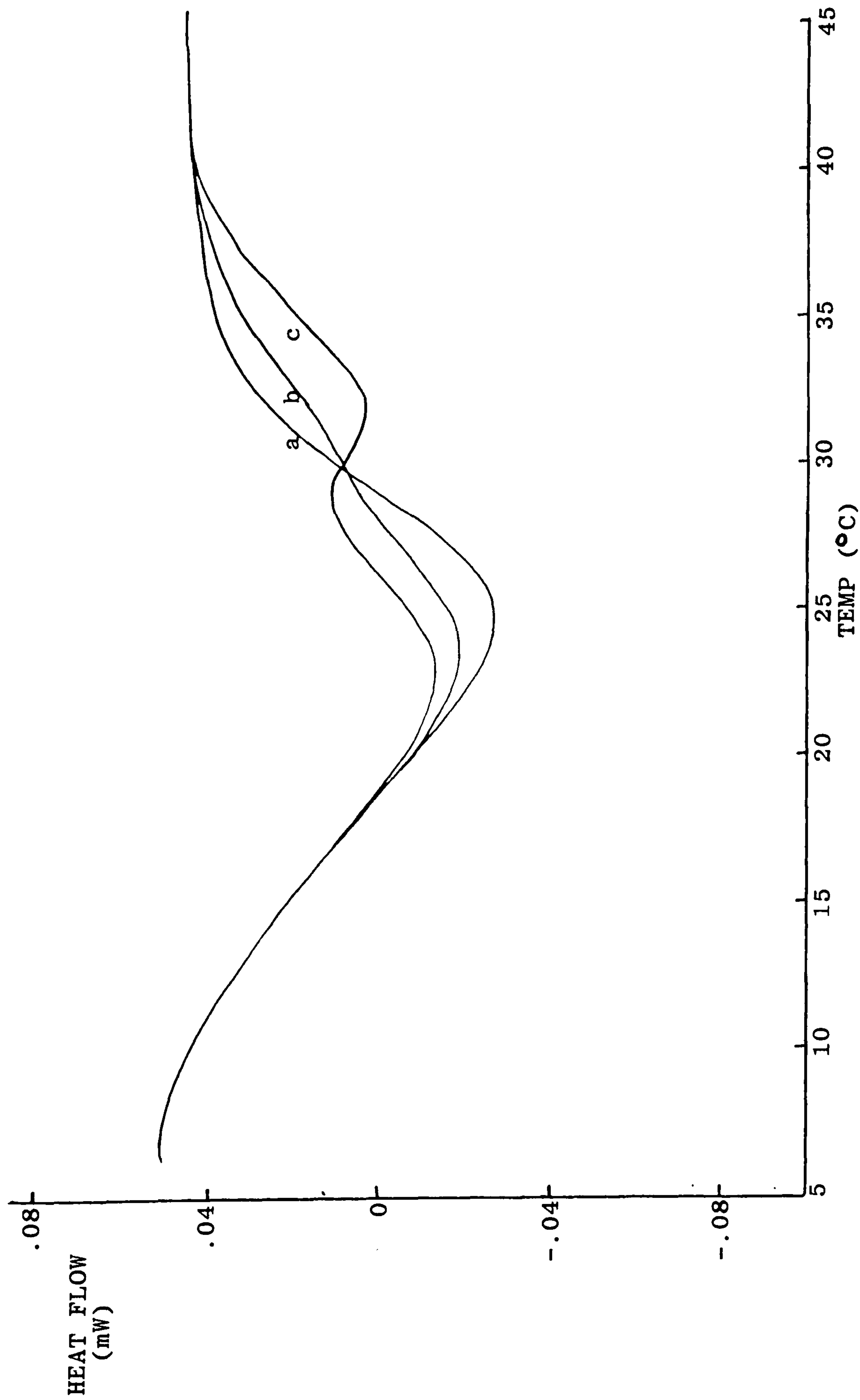


Figure 7.21. DSC melting endotherms of LO-1 gels ( $20 \text{ mg ml}^{-1}$ ) formed by cooling to  $25^\circ\text{C}$  for various lengths of time and then further cooling to  $5^\circ\text{C}$  for 16 hours: a) zero time at  $25^\circ\text{C}$ ; b) 8 hours at  $25^\circ\text{C}$ ; c) 35 hours at  $25^\circ\text{C}$ .

## 7.7. CONCLUSIONS

A Monte Carlo simulation program has been developed which is capable of reproducing most of the experimentally-observed phenomena associated with gelatin gelation and melting.

Initial attempts at simulation, assuming random nucleation of helices at any point within the chain and subsequent helix winding to the maximum geometric helix length, has been shown to be an unsatisfactory model. This is because the helices produced under such circumstances are too stable (i.e. too long) and as such are not affected by the temperature chosen for renaturation, giving helix fractions and melting points inconsistent with experimental values, irrespective of the renaturation temperature.

However, introduction of a parameter (theoretically associated with the likelihood of amino acid residues existing in the *cis* rather than the *trans* configuration) to allow multiple nucleations within the same chain, by limiting the helix lengths attainable, produced simulated results which were in excellent agreement with experimental results.

The program uses simple thermodynamics to assess the stabilities of helices generated, thereby enabling both melting curves and, to a certain extent, renaturation



curves to be simulated with the use of known experimental parameters in conjunction with 'sensible values' for other experimentally undetermined parameters.

From the simulation results a value of  $x$  (the number of units which cannot participate in bonding due to end effects) equal to 1 has been identified as the most appropriate value, which is also consistent with various proposed models for the collagen triple helix. Furthermore, the simulated results produce the best agreement with experiments for collagen melting temperatures of 37-38°C and PKINK (the lower limit of the equilibrium constant for the *trans-cis* isomerisation) in the range 0.14 to 0.17.

## CHAPTER 8: SUMMARY AND CONCLUSIONS

From this study of gelatin gelling and melting phenomena, numerous results of interest have arisen. The detailed examination of the melting behaviour presented in Chapter 5 clearly illustrates the critical importance of curing temperature in controlling the properties (i.e. gel strength, melting temperature) of the gels produced. Indeed, the melting behaviour of any gel produced is specific for the particular temperature at which it is formed. Curing at higher temperatures produces gels in which the triple helix junction zones are of increased thermal stability compared to those produced by curing at low temperature. This increased stability is attributed to an increase in the average length of the helical sequences formed at higher temperatures over those formed at lower temperatures, particularly as the experimental results gave no indication of other possible stabilisation mechanisms such as aggregation of helices or specific residue interactions within the helical structure. Furthermore, the melting temperatures corresponding to specific temperatures of gel formation were found to be approximately the same for all the gelatin samples investigated, a result anticipated from the proposal that stability is determined by helix length, as this would, to a first approximation, be independent of sample type.

Curing at high temperatures before cooling to lower temperatures results in gels with considerably higher storage moduli than those formed by curing only at low temperature,



even though the helix fraction and size of the endothermic melting peak are approximately the same for the two cooling regimes. This result appears to conflict with the proposal of longer helices causing increased melting points, as longer helices would of necessity imply fewer helices, which in turn might be expected to give lower values of rigidity. However, studies of the initial rate kinetics of the renaturation process (Chapter 6) showed two different mechanisms for conformational ordering. At low concentration the process was first order, consistent with a unimolecular reaction, whereas at higher concentrations, a second order process was found to be superimposed on the first order process. It is presumed that the first order reaction is a result of reverse folding of gelatin chains intramolecularly (a process which would not significantly alter the rigidity modulus) while the second order process, involving two chains and initiated at a  $\beta$  bend (or similar tight turn), is responsible for network formation. The maximum length of intermolecular helices is greater than that for intramolecular ordering (half and one third of the total chainlength, respectively), introducing a selective bias towards intermolecular association at higher temperatures, where only long helices are stable. Consequently, the network forming capabilities of the system would be enhanced by holding at higher temperatures, thus reconciling the initially conflicting observations discussed above.



A "Monte Carlo" simulation program based on the reaction scheme proposed from the kinetic results, was developed to simulate the events occurring in gelatin gelation and melting. The program uses simple thermodynamic criteria to assess the thermal stability of helices according to their length, thereby simulating the effect of temperature on the helix population. The results obtained during program development did not support the initial assumption that helix propagation proceeds to geometric limits. An alternative proposal that helix length is limited by the rate of *cis-trans* isomerisation of peptide bonds gave simulated renaturation curves of the same general shape as those obtained experimentally, with realistic values for the 'final helix fraction' at different temperatures. The melting profiles of these simulated helix populations showed excellent agreement with experimental results from optical rotation, and the bimodal melting behaviour observed by differential scanning calorimetry for samples held for a long period of time at some higher temperature before final aging at 5°C, were also reproduced in the computer model.

In conclusion, therefore, virtually all aspects of the experimental results could be reproduced using the simulation program, thereby validating the assumptions made within the program, and offering a reasonable interpretation of the events occurring during gelatin gelation and melting.

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APPENDIX 1. FORTRAN LISTING OF PROGRAM

```

      DIMENSION RL(50000),NC(50000),MATE(2,50000),ND(50000)
      DIMENSION END(50000),DEND(50000),F(7),R(7)
      DIMENSION RLH(3,50),IH(3),IHC(3)
      COMMON NST,RL,NC,MATE
      COMMON /COIL/XNUC,TMAX,DHICAL
      CHARACTER*15 FILE1
      DATA IYES/'Y'/
      IDIM=50000
      TOL=0.0005
      -----
      C-----
      C SEEDS "RAN" FUNCTION AND READS IN 'SAMPLE' CONDITIONS
      C-----
      10 WRITE(6,20)
      20 FORMAT(///,' GIVE ME A 5 FIGURE NUMBER PLEASE '$)
      READ(5,*)ISEED
      IP=2*ISEED+1
      WRITE(6,22)
      22 FORMAT(/,' WHAT VALUE OF XNUC ? '$)
      READ(5,*)XNUC
      WRITE(6,23)
      23 FORMAT(/,' WHAT VALUE FOR PKINK ? '$)
      READ(5,*) PKINK
      WRITE(6,24)
      24 FORMAT(/,' PROBABILITY OF OVERCOMING CIS KINK ? '$)
      READ(5,*) PTRANS
      WRITE(6,26)
      26 FORMAT(/,' TM FOR NATIVE COLLAGEN (C) ? '$)
      READ(5,*) TMAX
      WRITE(6,28)
      28 FORMAT(/,' ENTHALPY CHANGE PER TRIPEPTIDE (KJ/MOL) ? '$)
      READ(5,*) DHICAL
      WRITE(6,30)
      30 FORMAT(/,' REL. PROBABILITY OF INTERMOLECULAR ASSOCIATION? '$)
      READ(5,*)PINTER
      PIN=PINTER/(1.0+PINTER)
      WRITE(6,35)
      35 FORMAT(/,' NUMBER OF CHAINS ? '$)
      READ(5,*) NCT
      WRITE(6,40)
      40 FORMAT(/,' CHAINLENGTH DISTRIBUTION,MIN/MAX(RANGE 0 TO 1) ? '$)
      READ(5,*) RLMIN
      WRITE(6,42)
      42 FORMAT(/,' OUTPUT FILENAME ? '$)
      READ(5,44) FILE1
      44 FORMAT(A15)
      OPEN(UNIT=30,FILE=FILE1,STATUS='NEW')
      -----
      C-----
      C CALCULATES CHAINLENGTH DISTRIBUTION AND INITIALISES ARRAYS
      C-----
      50 M=4
      RLT=0.0
      DO 65 I=1,NCT
      55 P=RAN(IP)
      IF(P.LT.0.99999) GOTO 55
      RL(I)=1.0
      GOTO 60
      55 RL(I)=(ALOG10(P/(1.0-P))/M)+0.5
      IF(RL(I).LT.0.0) RL(I)=0.0
      IF(RL(I).GT.1.0) RL(I)=1.0
      60 RL(I)=RLMIN+(1.0-RLMIN)*RL(I)

```



```

      IF(RL(I).EQ.0.0) GOTO 50
65      RLT=RLT+RL(I)
      DLT=RLT
      NST=NCT
      NDT=NCT
      DO 70 I=1,NCT
      NC(I)=I
      MATE(1,I)=0
      MATE(2,I)=0
      IF(I.EQ.1) END(I)=RL(I)
      IF(I.GT.1) END(I)=END(I-1)+RL(I)
      ND(I)=I
70      DEND(I)=END(I)
      DO 75 I=1,7
75      R(I)=0.0
      WRITE(30,85)RLT
      WRITE(6,85)RLT
85      FORMAT (/, ' TOTAL CUMULATIVE CHAINLENGTH (RLT)= ',F10.4)
      WRITE(6,80)
80      FORMAT(/, ' TABLE OF CHAINLENGTHS ? '$)
      READ(5,90) IRES
90      FORMAT(A1)
      IF(IRES.NE.IYES) GOTO 100
      CALL LENGTH(NCT,RL,RMIN)
-----
C-----
C      READS IN RUNNING CONDITIONS AND PRINTS TABLE HEADING
C
100     WRITE(6,110)
110     FORMAT(/, ' TEMPERATURE (C)? '$)
      READ (5,*)TC
      TK=TC+273.2
      IT=0
      MAXOLD=0
115     WRITE(6,120)
120     FORMAT(/, ' NUMBER OF ITERATIONS,OR 0 FOR CONVERGENCE TEST ? '$)
      READ(5,*)MAXIT
      MAXIT=MAXIT+MAXOLD
      WRITE(6,122)
122     FORMAT(/, ' ITERATION INTERVAL FOR TABLE ? '$)
      READ(5,*) NTAB
      WRITE(6,125) MAXIT,TC,TMAX,XNUC,NCT,RLMIN,PINTER,PTRANS,
+      DHCAL,ISEED
      WRITE(30,125) MAXIT,TC,TMAX,XNUC,NCT,RLMIN,PINTER,PTRANS,
+      DHCAL,ISEED
125     FORMAT('1MAXIMUM NUMBER OF ITERATIONS ',21(' '),I7,
+      /' QUENCH TEMPERATURE (C) ',27(' '),F5.1,
+      /' TM FOR NATIVE COLLAGEN (C) ',23(' '),F5.1,
+      /' NUCLEATION LENGTH (XNUC) ',25(' '),F5.2,
+      /' NUMBER OF CHAINS ',33(' '),I5,
+      /' CHAINLENGTH DISTRIBUTION (MIN/MAX) ',15(' '),F5.2,
+      /' RELATIVE PROBABILITY OF INTERMOLECULAR ORDER ',5(' '),F6.2,
+      /' PROBABILITY OF OVERCOMING CIS KINK ',15(' '),F5.2,
+      /' ENTHALPY CHANGE PER TRIPEPTIDE (KJ/MOL) ',10(' '),F6.2,
+      /' SEED FOR RANDOM NUMBER GENERATOR ',17(' '),I6)
      WRITE(6,127)
      WRITE(30,127)
127     FORMAT('1ITERATION RESULTS')
      WRITE(6,130)
      WRITE(30,130)
130     FORMAT(/,25X,'AVERAGE NUMBER OF HELICAL          AVERAGE HELIX ')

```

```

+ , 'LENGTH', /, ' NUMBER OF      HELIX', 9X, 'SEQUENCES PER CHAIN', 6X
+ , '(RELATIVE TO LMAX OF 1000)', /, 3X, 'CYCLES  FRACTION', 5X
+ , 'TOTAL      INTRA      INTER      OVERALL      INTRA      INTER', /)

```

```

C-----
C      SELECTS SEQUENCE AND PROCESS
C

```

```

150  P=RAN(IP)
      P=P*RLT
      I=0
160  I=I+1
      IF(END(I).GE.P) GOTO 170
      GOTO 160
170  IF(MATE(1,I).NE.0) GOTO 400
      P=RAN(IP)
      IF(PIN.GT.P) GOTO 250

```

```

C-----
C      ORDERING WITHIN A SINGLE CHAIN SEQUENCE
C

```

```

C      NUCLEATES HELIX AND TESTS FOR STABILITY
C

```

```

180  P=RAN(IP)
      X=P*RL(I)
      P=RAN(IP)
      Y=P*RL(I)
      NCASE=1
      IF(X.LE.Y.AND.X.GT.((Y-X)/2.0)) NCASE=2
      IF(X.GT.Y.AND.(RL(I)-X).LE.((X-Y)/2.0)) NCASE=3
      IF(X.GT.Y.AND.(RL(I)-X).GT.((X-Y)/2.0)) NCASE=4
      IF(NCASE.EQ.1) HL=X
      IF(NCASE.EQ.2) HL=(Y-X)/2.0
      IF(NCASE.EQ.3) HL=RL(I)-X
      IF(NCASE.EQ.4) HL=(X-Y)/2.0
      RN=HL*1000.0
      LITTLE=0
      RNCIS=0.0
165  P=RAN(IP)
      EXTRA=ALOG(P)/4LOG(1.0-PKINK)
      RNCIS=RNCIS+EXTRA
      IF(RNCIS.GT.RN) GOTO 185
      P=RAN(IP)
      IF(PTRANS.GT.P) GOTO 165
      LITTLE=1
      HL=RNCIS/1000.0
      RN=RNCIS
185  PUN=PCOIL(RN,TK)
      P=RAN(IP)
      IF(PUN.GT.P) GOTO 500

```

```

C-----
C      SUCCESSFUL INTRAMOLECULAR NUCLEATION
C

```

```

      IF(LITTLE.EQ.1) GOTO 225
      CALL OPEN(I,4)
      DO 190 K=1,4
      MATE(1,(I-K))=0
      MATE(2,(I-K))=0
190  NC(I-K)=NC(I)
      IF(NCASE.EQ.2) GOTO 200
      IF(NCASE.EQ.3) GOTO 210
      IF(NCASE.EQ.4) GOTO 220
      RL(I-2)=Y-3.0*X

```

```

      RL(I)=RL(I)-Y
      K=I-1
      L=I-3
      M=I-4
      GOTO 230
200    RL(I-4)=((3.0*X)-Y)/2.0
      RL(I)=RL(I)-Y
      K=I-1
      L=I-2
      M=I-3
      GOTO 230
210    RL(I-4)=Y
      RL(I-2)=(3.0*X)-(2.0*RL(I))-Y
      K=I
      L=I-1
      M=I-3
      GOTO 230
220    RL(I-4)=Y
      RL(I)=RL(I)-(((3.0*X)-Y)/2.0)
      K=I-1
      L=I-2
      M=I-3
      GOTO 230
225    CALL OPEN(I,5)
      DO 235 K=1,5
      MATE(1,(I-K))=0
      MATE(2,(I-K))=0
235    NC(I-K)=NC(I)
      IF(X.GT.Y) GOTO 240
      RL(I-5)=X-HL
      RL(I-2)=Y-X-2.0*HL
      RL(I)=RL(I)-Y
      K=I-1
      L=I-3
      M=I-4
      GOTO 230
240    RL(I-5)=Y
      RL(I-3)=X-Y-2.0*HL
      RL(I)=RL(I)-X-HL
      K=I-1
      L=I-2
      M=I-4
      GOTO 230

```

---

```

C -----
C      CREATES NEW HELICAL SEQUENCES
C

```

```

230    RL(K)=HL
      RL(L)=HL
      RL(M)=HL
      MATE(1,K)=L
      MATE(2,K)=M
      MATE(1,L)=K
      MATE(2,L)=M
      MATE(1,M)=K
      MATE(2,M)=L
      GOTO 450

```

---

```

C -----
C      INTERMOLECULAR ORDERING
C
250    IF(NDT.EQ.1) GOTO 180

```



```

260 P=RAN(IP)
    P=P*DLT
    K=0
270 K=K+1
    IF(DEND(K).LT.P) GOTO 270
    IF(ND(K).EQ.I) GOTO 260
    J=ND(K)
C-----
C      FOUND A PARTNER
C
C      NOW NUCLEATE HELIX AND TEST FOR STABILITY
C
    P=RAN(IP)
    X=P*RL(I)
    P=RAN(IP)
    Y=P*RL(J)
    NCASE=5
    IF(Y.LT.X.AND.Y.LT.(RL(I)-X)) GOTO 280
    NCASE=6
    IF(X.GT.(RL(I)-X)) NCASE=7
280 IF(NCASE.EQ.5) HL=Y
    IF(NCASE.EQ.6) HL=X
    IF(NCASE.EQ.7) HL=RL(I)-X
    RN=HL*1000.0
    LITTLE=0
    RNCIS=0.0
265 P=RAN(IP)
    EXTRA=ALOG(P)/ALOG(1.0-PKINK)
    RNCIS=RNCIS+EXTRA
    IF(RNCIS.GT.RN) GOTO 285
    P=RAN(IP)
    IF(PTRANS.GT.P) GOTO 265
    LITTLE=1
    HL=RNCIS/1000.0
    RN=RNCIS
285 PUN=PCOIL(RN,TK)
    P=RAN(IP)
    IF(PUN.GT.P) GOTO 500
C-----
C      SUCCESSFUL INTERMOLECULAR NUCLEATION
C
C
    JBIG=0
    IF(J.GT.I) JBIG=1
    IF(LITTLE.EQ.0) GOTO 295
    CALL OPEN(I,3)
    IF(JBIG.EQ.1) J=J+3
    IF(JBIG.EQ.0) I=I+2
    CALL OPEN(J,2)
    DO 305 L=1,2
    MATE(L,(I-3))=0
305 MATE(L,(J-2))=0
    NC(I-1)=NC(I)
    NC(I-2)=NC(I)
    NC(I-3)=NC(I)
    NC(J-1)=NC(J)
    NC(J-2)=NC(J)
    RL(I)=RL(I)-X-HL
    RL(I-3)=X-HL
    RL(J)=RL(J)-Y
    RL(J-2)=Y-HL

```

```

      K=I-1
      L=I-2
      M=J-1
      GOTO 230
295  IF(NCASE.NE.5) GOTO 310
      CALL OPEN(I,3)
      IF(JBIG.EQ.1) J=J+3
      IF(JBIG.EQ.0) I=I+1
      CALL OPEN(J,1)
290  MATE(1,(I-3))=0
      MATE(2,(I-3))=0
      DO 300 K=1,3
300  NC(I-K)=NC(I)
      RL(I-3)=X-Y
      RL(I)=RL(I)-X-Y
      RL(J)=RL(J)-Y
      K=I-2
      L=I-1
      M=J-1
      GOTO 230
310  CALL OPEN(I,2)
      IF(JBIG.EQ.1) J=J+2
      IF(JBIG.EQ.0) I=I+2
      CALL OPEN(J,2)
330  DO 350 K=1,2
      DO 340 L=1,2
      MATE(L,(I-K))=MATE(L,I)
340  MATE(L,(J-K))=MATE(L,J)
      NC(I-K)=NC(I)
350  NC(J-K)=NC(J)
      IF(NCASE.EQ.7) GOTO 360
      RL(I)=RL(I)-2.0*X
      RL(J-2)=Y-X
      RL(J)=RL(J)-Y
      K=I-2
      L=I-1
      M=J-1
      GOTO 230
360  RL(I-2)=(2.0*X)-RL(I)
      RL(J-2)=Y+X-RL(I)
      RL(J)=RL(J)-Y
      K=I-1
      L=I
      M=J-1
      GOTO 230

```

```

C -----
C      HELIX UNWINDING
C
400  P=RAN(IP)
      RN=RL(I)*1000.0
      PUN=PCOIL(RN,TK)
      IF(PUN.LT.P) GOTO 500
      IHC(1)=NC(I)
      IHC(2)=NC(MATE(1,I))
      IHC(3)=NC(MATE(2,I))
      IH(1)=I
      IH(2)=MATE(1,I)
      IH(3)=MATE(2,I)
      DO 410 M=1,3
      DO 410 L=1,2

```

```

410  MATE(L,IH(M))=0
      CALL MERGE(IHC(1))
      CALL MERGE(IHC(2))
      CALL MERGE(IHC(3))
C-----
C      UPDATES END AND DEND ARRAYS
C
450  NDT=0
      DLT=0.0
      RLCUM=0.0
      DO 460 I=1,NST
          RLCUM=RLCUM+RL(I)
          END(I)=RLCUM
          IF(MATE(1,I).NE.0)GOTO 460
          NDT=NDT+1
          ND(NDT)=I
          DLT=DLT+RL(I)
          DEND(NDT)=DLT
460  CONTINUE
      ERR=(RLCUM-RLT)/RLT
      IF(ERR.LT.0.00001) GOTO 500
      WRITE (5,470)
470  FORMAT(//,' SERIOUS ROUNDING ERROR IN SEQUENCE BOUNDARIES')
C-----
C      END OF CYCLE
C
500  IT=IT+1
      ISTOP=0
      IF(IT.LT.MAXIT.OR.MAXIT.EQ.0) GOTO 510
      ISTOP=1
      GOTO 530
510  IF(NST.LT.(IDIM-6)) GOTO 520
      ISTOP=1
      GOTO 530
520  IF(MOD(IT,NTAB).EQ.0) GOTO 530
      GOTO 150
C-----
C      CALCULATES HELIX PARAMETERS AND TESTS FOR CONVERGENCE
C
530  NH1=0
      NH2=0
      HL1=0.0
      HL2=0.0
      DO 550 I=1,NST
          IF(MATE(1,I).EQ.0) GOTO 550
          L=NC(I)
          J=NC(MATE(1,I))
          K=NC(MATE(2,I))
          IF(L.EQ.J.AND.L.EQ.K) GOTO 540
          NH2=NH2+1
          HL2=HL2+RL(I)
          GOTO 550
540  NH1=NH1+1
      HL1=HL1+RL(I)
550  CONTINUE
      DO 552 NF=1,7
552  F(NF)=0.0
      RH1=NH1
      RH2=NH2

```



```

      F(1)=(HL1+HL2)/RLT
      F(2)=(RH1+RH2)/NCT
      F(3)=RH1/NCT
      F(4)=RH2/NCT
      IF((NH1+NH2).EQ.0) GOTO 558
      F(5)=((HL1+HL2)/(NH1+NH2))*1000.0
      IF(NH1.EQ.0) GOTO 556
      F(6)=(HL1/NH1)*1000.0
556   IF(NH2.EQ.0) GOTO 558
      F(7)=(HL2/NH2)*1000.0
558   IF (ISTOP.EQ.1) GOTO 600
C-----
C      PRINTS HELIX PARAMETERS
C
600   WRITE(6,610)IT,(F(I),I=1,7)
      WRITE(30,610)IT,(F(I),I=1,7)
610   FORMAT(I10,F10.5,3F10.3,3F10.1)
      IF(ISTOP.EQ.0) GOTO 150
C-----
C      PRINTS OUT LENGTH DISTRIBUTION
C
      WRITE(6,620)
620   FORMAT('/', ' LENGTH DISTRIBUTION?  '$)
      READ(5,90)IRES
      IF(IRES.NE.IYES) GOTO 900
      WRITE(6,630)
      WRITE(30,630)
630   FORMAT('/', '1DISTRIBUTION OF HELIX LENGTHS',/,
+      ' (RELATIVE TO MAXIMUM CHAIN LENGTH OF 1000)',/,
+      15X, 'HELIX FRACTION * 1000',/,
+      '      LENGTH      TOTAL      INTRA      INTER',/)
      DO 640 I=1,3
      DO 640 J=1,40
640   RLH(I,J)=0.0
      DO 650 I=1,NST
      IF(MATE(1,I).EQ.0) GOTO 650
      M1=MATE(1,I)
      M2=MATE(2,I)
      J=RL(I)*100.0+0.5
      IF(J.LT.1) J=1
      IF(J.GT.40) J=40
      FAC=1000.0/RLT
      RLH(1,J)=RLH(1,J)+RL(I)*FAC
      IF(NC(I).EQ.NC(M1).AND.NC(I).EQ.NC(M2)) GOTO 645
      RLH(3,J)=RLH(3,J)+RL(I)*FAC
      GOTO 650
645   RLH(2,J)=RLH(2,J)+RL(I)*FAC
650   CONTINUE
      DO 660 J=1,40
      LEN=10*J
      DO 655 I=1,3
655   RLH(I,J)=RLH(I,J)+RLH(I,(J+1))
      WRITE(6,670) LEN,(RLH(I,J),I=1,3)
660   WRITE (30,670) LEN,(RLH(I,J),I=1,3)
670   FORMAT(I10,3F10.2)
      GOTO 900
C-----
C      PRINTS STORAGE ARRAYS
C
700   WRITE(6,710)

```

```

710  FORMAT(//, ' TABLE OF CHAIN SEQUENCES ? '$)
      READ(5,90)IRES
      IF(IRES.NE.IYES) GOTO 800
      WRITE(30,720)
      WRITE(6,720)
720  +  FORMAT(////, '1 SEQUENCE          CHAIN    RL*1000',
      +  '    MATE(1)    MATE(2)    TOTAL LENGTH',/)
      DO 740 I=1,NST
      SL=RL(I)*1000.0
      IF (I.EQ.1) GOTO 739
      IF (NC(I).NE.NC(I-1)) WRITE(6,730)
      IF (NC(I).NE.NC(I-1)) WRITE(30,730)
730  FORMAT(65(' '))
739  WRITE (5,750)I,NC(I),SL,MATE(1,I),MATE(2,I),END(I)
740  WRITE (30,750)I,NC(I),SL,MATE(1,I),MATE(2,I),END(I)
750  FORMAT(2I10,F10.2,2I10,F15.4)
      WRITE(30,760)
760  +  FORMAT(////, '1',19X,'DISORDERED SEQUENCES',/,12X,
      +  'SEQUENCE          CHAIN    RL*1000    CUMULATIVE LENGTH',/)
      DO 770 J=1,NDT
      I=ND(J)
      SL=RL(I)*1000.0
770  WRITE(30,780)J,I,NC(I),SL,DEND(J)
780  FORMAT(3I10,F10.2,F15.4)
-----
C
C      CHANGES UNWINDING PARAMETERS, STARTS AGAIN OR STOPS
C
800  WRITE(6,810)
810  FORMAT(//, ' ANOTHER RUN ? '$)
      READ(5,90)IRES
      IF(IRES.NE.IYES) STOP
      WRITE(6,820)
820  FORMAT(/, ' START AGAIN ? '$)
      READ(5,90)IRES
      IF(IRES.EQ.IYES) GOTO 10
      WRITE(6,830)
830  FORMAT(/, ' SAME TEMPERATURE ? '$)
      READ(5,90) IRES
      IF(IRES.NE.IYES) GOTO 100
      MAXOLD=MAXIT
      GOTO 115
-----
C
C      PRINTS OUT MELTING PROFILE (DSC SIMULATION)
C
900  WRITE(6,910)
910  FORMAT(//, ' MELTING PROFILE? '$)
      READ(5,90)IRES
      IF(IRES.NE.IYES)GOTO 700
      WRITE(6,920)
      WRITE(30,920)
920  +  FORMAT(//, '1HELIX MELTING PROFILE',//,
      +  '9X, 'HELIX FRACTION    DELTA HF',/,
      +  ' T(C)          (PERCENT)          (*1000)',//)
      FORMER=F(1)*1000.0
      DO 940 J=1,50
      TOT=0.0
      DO 930 I=1,NST
      IF(MATE(1,I).EQ.0)GOTO 930
      RES=RL(I)*1000.0
      TEMP=J+273.2

```

```

PUN=PCOIL(RES,TEMP)
TOT=TOT+(RES*PUN/RLT)
930 CONTINUE
HELIX=((F(1)*1000.0)-TOT)/10.0
DELTA=FORMER-(HELIX*10.0)
FORMER=HELIX*10.0
WRITE(6,950) J,HELIX,DELTA
940 WRITE(30,950) J,HELIX,DELTA
950 FORMAT(I3,2F15.2)
GOTO 700
END

C -----
C
SUBROUTINE LENGTH(NCT,RL,RLMIN)
C
C CONSTRUCTS TABLE OF PRIMARY CHAINLENGTHS
C
C
C DIMENSION RL(50000),NL(101)
C WRITE(30,10)
C WRITE(6,10)
10 FORMAT(/, '1DISTRIBUTION OF CHAINLENGTHS ',/,
+ ' (RELATIVE TO MAXIMUM LENGTH OF 1000)',/,
+ ' LENGTH NUMBER',/)
RANGE=1.0-RLMIN
NP=100
DIFF=RANGE/NP
DO 20 I=1,NP
20 NL(I)=0
DO 30 I=1,NCT
J=(RL(I)-RLMIN)/DIFF+0.5
IF(J.LT.1) J=1
IF(J.GT.NP) J=NP
30 NL(J)=NL(J)+1
DO 40 I=1,NP
LEN=(RLMIN+(I*DIFF))*1000.0+0.5
WRITE(30,50) LEN,NL(I)
40 WRITE(6,50) LEN,NL(I)
50 FORMAT(2I10)
RETURN
END

C -----
C
SUBROUTINE OPEN(N,M)
C
C CREATES M VACANT ARRAY ELEMENTS IMMEDIATELY BEFORE ELEMENT N
C
C DIMENSION RL(50000),NC(50000),MATE(2,50000)
C COMMON NST,RL,NC,MATE
C
C DO 10 J=1,NST
C DO 10 I=1,2
C IF(MATE(I,J).GE.N) MATE(I,J)=MATE(I,J)+M
10 CONTINUE
I=NST
20 IF(I.LT.N) GOTO 30
RL(I+M)=RL(I)
NC(I+M)=NC(I)
MATE(1,(I+M))=MATE(1,I)
MATE(2,(I+M))=MATE(2,I)
I=I-1
GOTO 20

```



```

30   NST=NST+M
      N=N+M
      RETURN
      END
C-----
      SUBROUTINE MERGE(N)
C-----
C-----
C-----
      COMBINES ADJACENT DISORDERED SEQUENCES IN CHAIN N
C-----
      DIMENSION RL(50000),NC(50000),MATE(2,50000)
      COMMON NST,RL,NC,MATE
      I=0
      5   I=I+1
          IF(NC(I).EQ.N) GOTO 15
          GOTO 5
      10  I=I+1
          IF(NC(I).NE.N) RETURN
      15  IF(MATE(1,I).NE.0) GOTO 10
      20  IF((I+1).GT.NST.OR.NC(I+1).NE.N) RETURN
          IF(MATE(1,(I+1)).EQ.0) GOTO 25
          I=I+1
          GOTO 10
      25  RL(I)=RL(I)+RL(I+1)
          NST=NST-1
          NDT=NDT-1
          DO 30 K=(I+1),NST
              NC(K)=NC(K+1)
              RL(K)=RL(K+1)
              MATE(1,K)=MATE(1,(K+1))
      30  MATE(2,K)=MATE(2,(K+1))
          DO 40 K=1,2
              DO 40 J=1,NST
                  IF(MATE(K,J).GT.(I+1)) MATE(K,J)=MATE(K,J)-1
      40  CONTINUE
          GOTO 20
      END
C-----
C-----
C-----
      PROBABILITY OF A HELIX OF LENGTH RN UNWINDING AT T(K)
C-----
      FUNCTION PCOIL(RN,T)
      COMMON /COIL/X,TMAX,DHCL
      TMD=((273.2+TMAX)*1000.0)/(1000.0-X)
      DHK=DHCL*1000.0
      GASCON=3.314
      PCOIL=1.0
      IF(RN.LE.X) RETURN
      TM=(RN-X)*TMD/RN
      RLK=RN*DHK*(TM-T)/(GASCON*T*TM)
      IF(RLK.GT.15.0) GOTO 10
      RK=EXP(RLK)
      PCOIL=1.0/(RK+1.0)
      RETURN
      10  PCOIL=0.0
          RETURN
          END

```

## APPENDIX 2

## MELTING BEHAVIOUR OF GELATIN GELS: ORIGIN AND CONTROL

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## ABSTRACT

Gelatin samples held at moderate temperatures (15 - 30°C) before quenching to low temperature (5°C) give stronger gels than those cooled directly to 5°C and show progressive development of a gel fraction of enhanced thermal stability, leading eventually to bimodal melting. We attribute this behaviour to selective formation at the holding temperature of helices longer than those formed at lower temperature, and present evidence from 'initial slope' kinetics and Monte Carlo simulation to suggest that the higher-melting species are predominantly functional, inter-molecular helices rather than 'wasted' intramolecular double-hairpin structures.

## INTRODUCTION

It is well known in the industry that the thermal history of gelatin gels can have a profound effect on their physical properties (1,2). In particular, gels held for some time at a moderate temperature before final quenching to a lower temperature are more rigid than those quenched directly to the lower temperature (3,4). We now report a detailed study of this behaviour, and offer an interpretation that may have much wider implications for understanding and controlling the formation and melting of gelatin gels.

## MATERIALS AND METHODS

The gelatin used was a first-extract lamed ossein sample kindly supplied by Rousselot. Solutions were prepared at 45°C, and sodium azide (0.02%) was incorporated to inhibit bacteria. Differential scanning calorimetry (DSC) studies were carried out on a Setaram microcalorimeter using a sample volume of 1ml and a scan rate of 0.1 degrees per minute. Optical rotation was measured at 365 nm on a Perkin-Elmer 241 polarimeter using thermostatted cells of pathlength 1 cm or 10 cm as appropriate. The fraction of residues in the ordered, helical conformation (fH) was calculated using the observed optical rotation of the disordered sol state (above 40°C) and the literature value for fully-ordered native collagen (5). Gel rigidity (storage

modulus, G') was measured at a frequency of 0.5Hz on a Sangamo Viscoelastic Analyser using cone and plate geometry of cone angle 2 degrees and diameter 5 cm.

## THERMAL HISTORY AND GEL MELTING

Figure 1 shows DSC melting endotherms for the same gelatin solution gelled under different thermal conditions. For each family of curves illustrated, the 'reference' state is a gel prepared by direct quenching of the hot (45°C) solution to a fixed temperature of 5°C for 16 hours, by which time further conformational ordering, as judged by optical rotation, is negligible over the time period of the DSC measurement (~7 hours). The other traces in each family show the effect of holding the sample for varying times at a higher temperature ( $T_0$ ) before again cooling to 5°C for 16h. In each case, as the length of time at the holding temperature is increased, the melting endotherms show progressive development of a higher-melting peak and eventually assume a distinct bimodal character, with the families of curves for each holding temperature crossing at a single isosbestic point. In all cases, however, the overall enthalpy change is essentially constant ( $\approx 3.2 \text{ kJ mol}^{-1}$ ) and the net helix fraction at 5°C is also virtually unchanged (at ~75%).

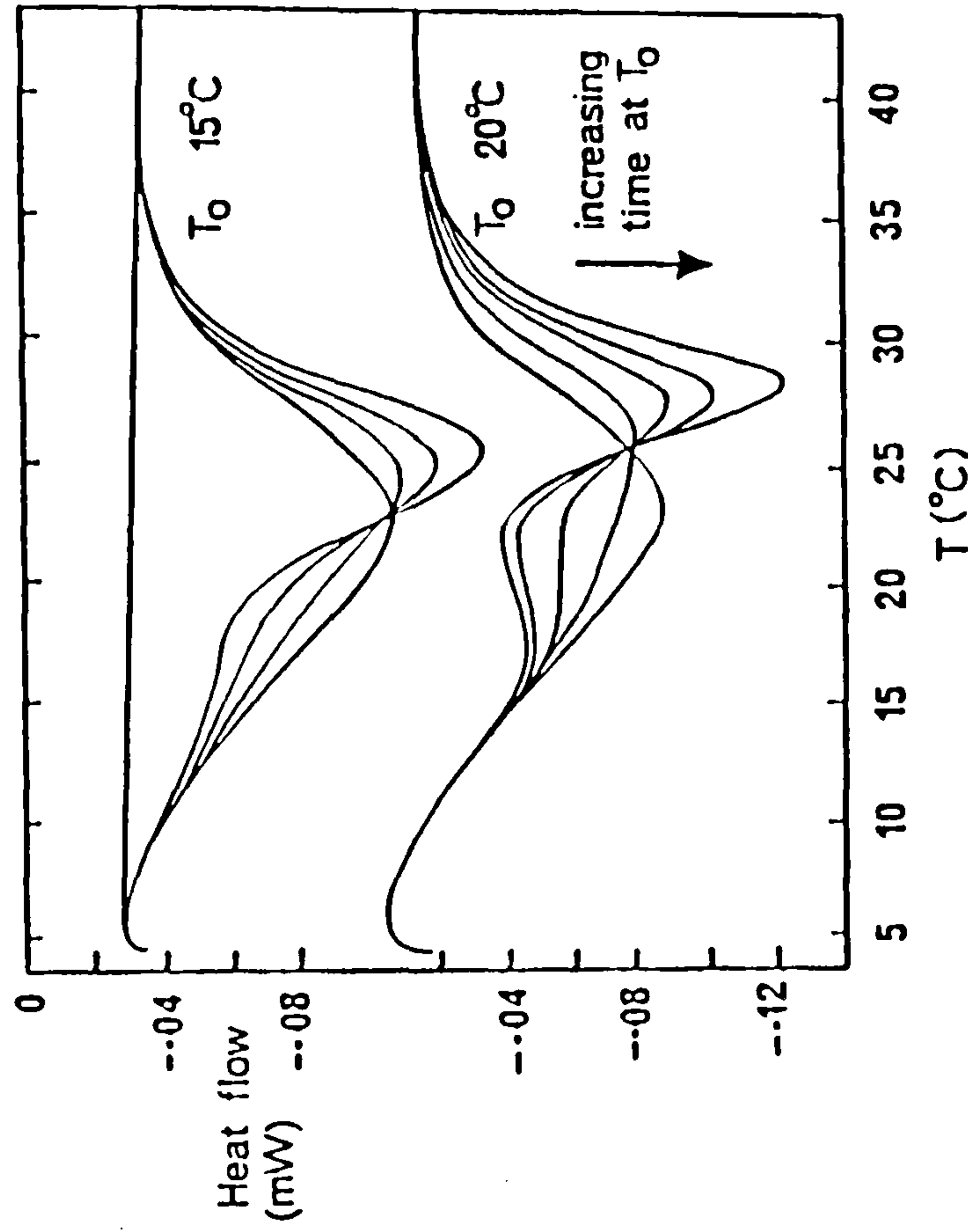


Figure 1. DSC melting endotherms for gels (20 mg ml<sup>-1</sup>) held (upper curves) at 15°C for 0, 1.5, 7 and 24 hours and (lower curves) at 20°C for 0, 2, 7, 24 and 72 hours before final quenching for 16 hours at 5°C in all cases. Similar families of curves were obtained using holding temperatures of 25°C and 30°C.



As illustrated in Fig. 2a, however, the rigidity ( $G'$ ) of gels held at higher temperature before final aging at  $5^\circ\text{C}$  is higher than that of the same sample cooled directly to  $5^\circ\text{C}$ , and remains higher throughout the melting process. In the case of samples held for sufficiently long at higher temperature to give obvious bimodal melting behaviour by DSC, the temperature-course of loss of rigidity on heating ( $dG'/dT$ ) also shows bimodal character, (Fig. 2b) with peaks that correspond closely to the DSC minima.

To investigate this behaviour further we have used optical rotation (a) to monitor the temperature-course of helix melting (i.e. local conformational change). As illustrated in Fig. 3 the rate of decrease in helix fraction with increasing temperature ( $d\alpha/dT$ ) is closely superimposable on the overall melting profile from DSC, both for samples quenched directly to  $5^\circ\text{C}$  and for those first held at higher temperature ( $T_0$ ) to give bimodal melting. Figure 3 also shows the temperature-course of helix melting when the sample is held for the same length of time at  $T_0$ , but then melted directly from this temperature without first cooling to  $5^\circ\text{C}$ . Under these conditions the temperature-dependence of  $d\alpha/dT$  is unimodal, joins the bimodal melting-curve at the isosbestic point illustrated in Fig. 1, and follows the same temperature-course thereafter.

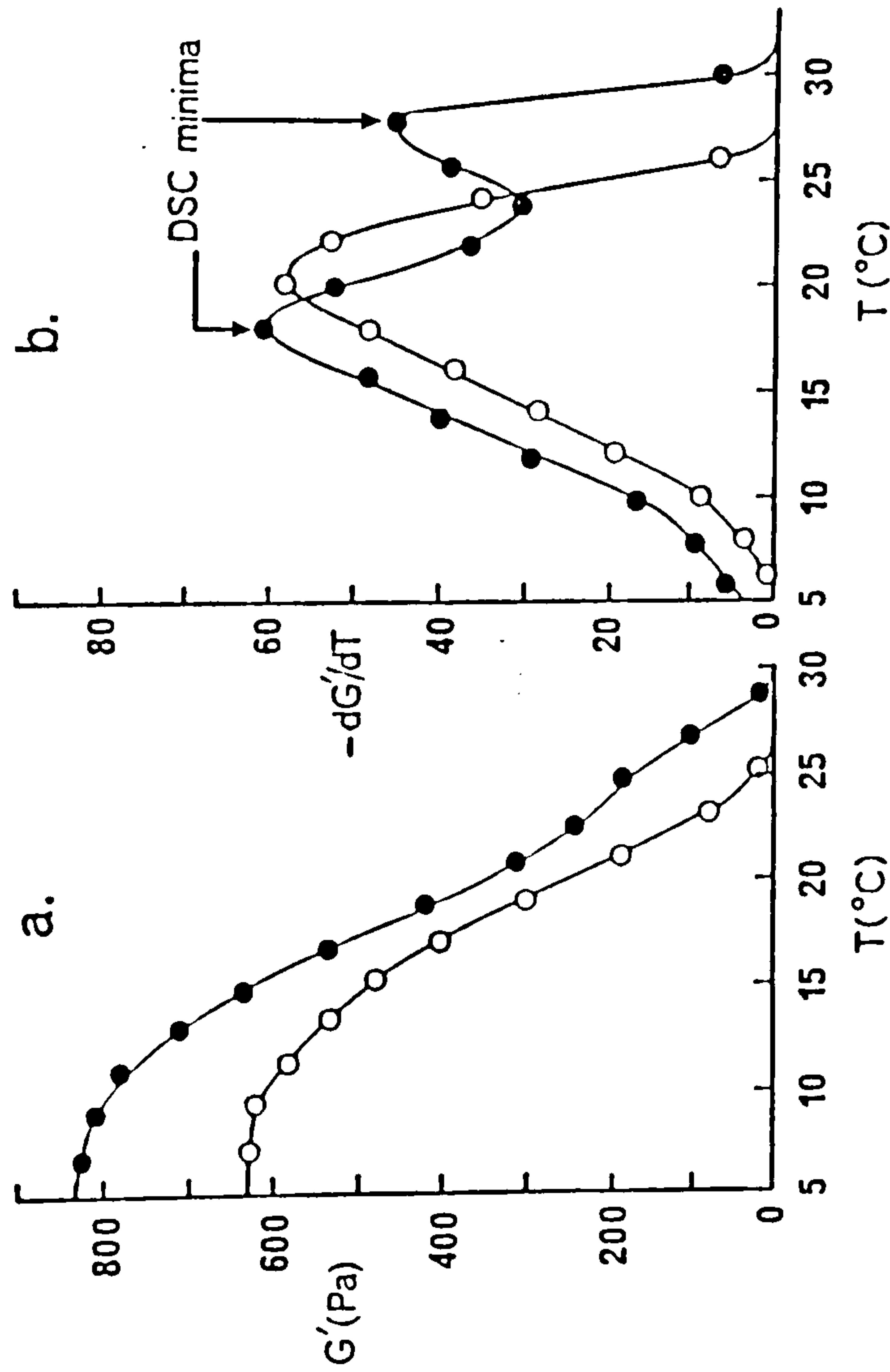


Figure 2. Temperature-dependence of rigidity ( $G'$ , 0.5 Hz) for gels ( $20\text{ mg ml}^{-1}$ ) quenched to  $5^\circ\text{C}$  for 16 hours, either directly (○) or after holding for 24 hours at  $20^\circ\text{C}$  (●); the final helix fraction is virtually identical in both cases ( $f_H = 0.75$ ). a) absolute values of  $G'$ , b) rate of change of  $G'$  with increasing temperature ( $dG'/dT$ ).

It therefore appears that the bimodal melting behaviour characterised independently by DSC, optical rotation and gel rigidity has its origin in the selective formation at  $T_0$  of a population of helices of greater thermal stability than those formed at lower temperature ( $5^\circ\text{C}$ ), which then remains virtually unchanged when the temperature is subsequently decreased. Quantitatively, the fractional amount by which the mid-point melting temperature ( $T_m$ ) exceeds the temperature at which the helices are formed ( $T_0$ ) decreases linearly with increasing  $T_0$  (Fig. 4a) and extrapolates to zero at  $T_0 = 36^\circ\text{C}$ , which probably corresponds to the melting point of the parent collagen (6). At each value of  $T_0$  the helix fraction increases linearly with the logarithm of holding time throughout the timescale of likely practical importance, as illustrated in Fig. 4b. Operationally, therefore, we can predict both the amount ( $f_H$ ) and thermal stability ( $T_m$ ) of the high-melting species formed after a known time at a specific holding temperature ( $T_0$ ). The origin of the enhanced stability, however, is less clear-cut.

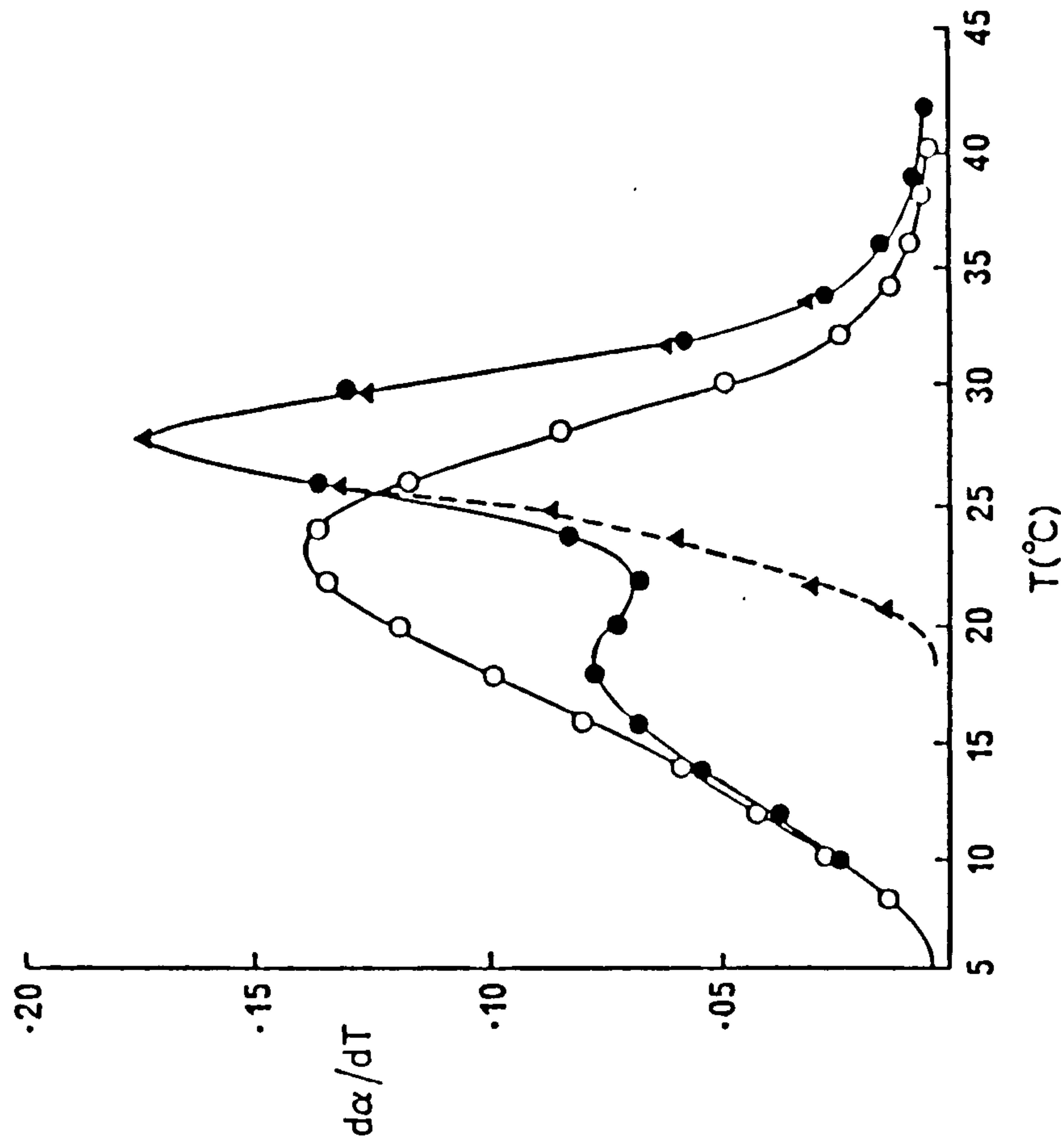


Figure 3. Thermal melting of gels ( $20\text{ mg ml}^{-1}$ ) quenched to  $5^\circ\text{C}$  for 16 hours directly (○) or after holding for 24 hours at  $20^\circ\text{C}$  (●); symbols show local conformational change by optical rotation ( $d\alpha/dT$ ) and solid lines show overall enthalpy change (Fig. 1) by DSC. Also shown (---) is the optical rotation change for the same sample melted directly after holding at  $20^\circ\text{C}$  for 24 hours.



### 'INITIAL SLOPE' KINETICS

There have been numerous studies of the dynamics of conformational ordering in collagen/gelatin systems, and many different and often conflicting, mechanisms have been proposed. (8). It is well established, however, that the kinetics of the sustained, gradual increase in helix fraction (and gel strength) at long times after quenching are particularly complex. In the very early stages of reaction, however, before the fraction of disordered sequences has decreased significantly from 100%, helix content increases linearly with time, and thus the initial slope of reaction progress curves such as that shown in Fig. 5 provides a direct measure of the rate of helix formation under these simple conditions. By using known changes in UV absorption to monitor helix formation at low concentrations and optical rotation at higher concentrations (9), we have determined initial reaction rates over a very wide range of gelatin concentrations ( $0.05 - 32 \text{ mg ml}^{-1}$ ).

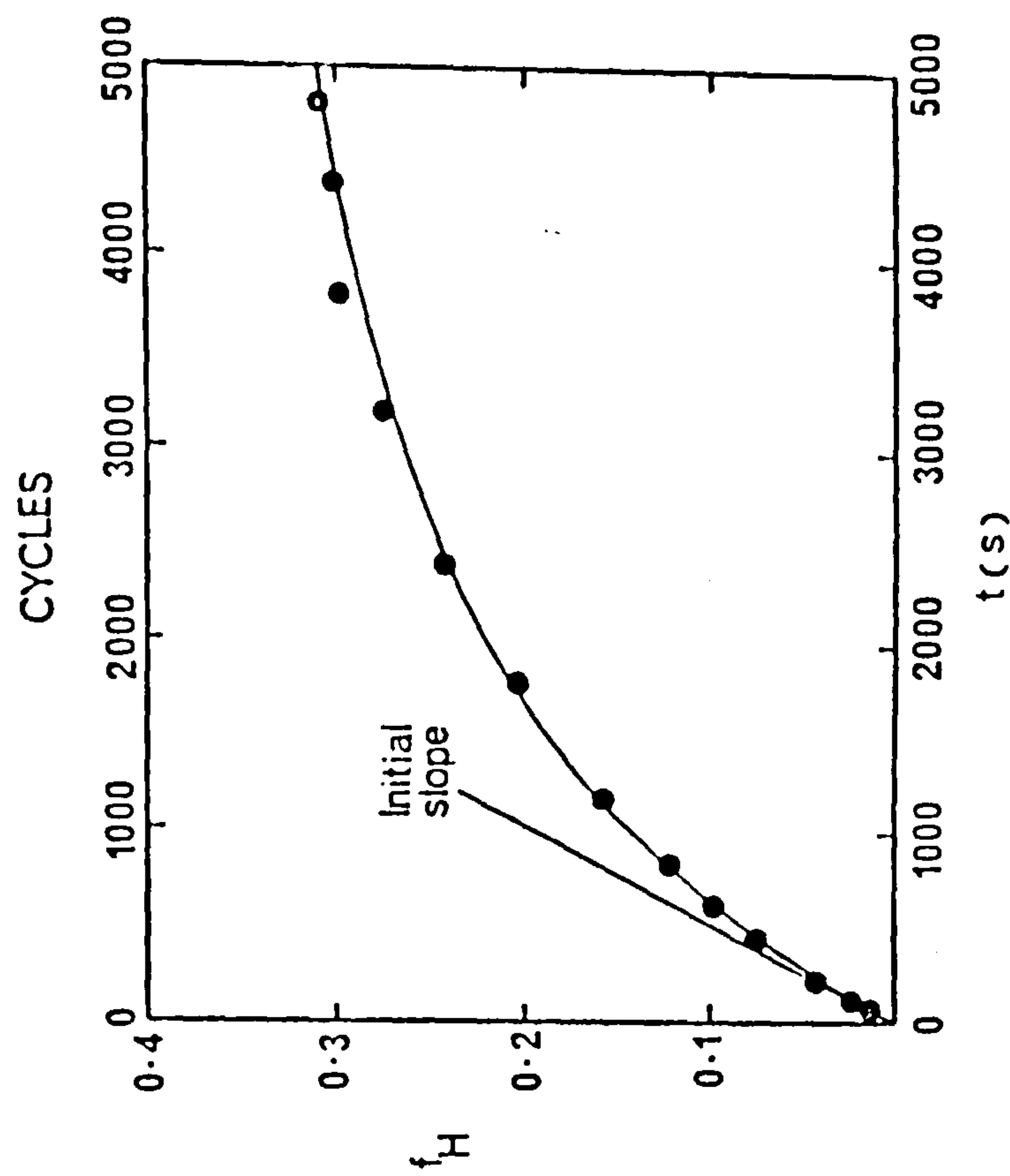


Figure 5. Comparison of observed reaction progress curve (solid line) for helix formation in gelatin ( $2 \text{ mg ml}^{-1}$ ;  $10^\circ\text{C}$ ) with values of helix fraction from the Monte-Carlo simulation described in the text, using  $A = 5$ ,  $B = 11.5$  and  $p(\text{inter}) = 0.5$  (i.e. equal probability of inter- and intra-molecular reaction). Determination of rate of conformational ordering in the early stages of reaction using the initial slope (S) of the reaction progress curve is also illustrated.

The close superposition (Fig. 3) of the temperature-course of the enthalpy change monitored by DSC and the conformational change by optical rotation argues against stabilisation of helices by aggregation unless the enthalpy change associated with the aggregation was undetectably small. A more likely interpretation is that the minimum critical sequence length for helix formation increases with increasing temperature, so that the helices formed at the higher temperature ( $T_0$ ) are longer than those formed on subsequent cooling and therefore melt at higher temperature (7). However, since holding at higher temperature has little if any effect on the final overall helix content, longer helices would of necessity imply fewer helices and therefore, at first sight, a weaker network rather than the more rigid structure observed experimentally (e.g. Fig. 2). We now present evidence from other lines of investigation which offers a unifying interpretation of these apparently conflicting observations.

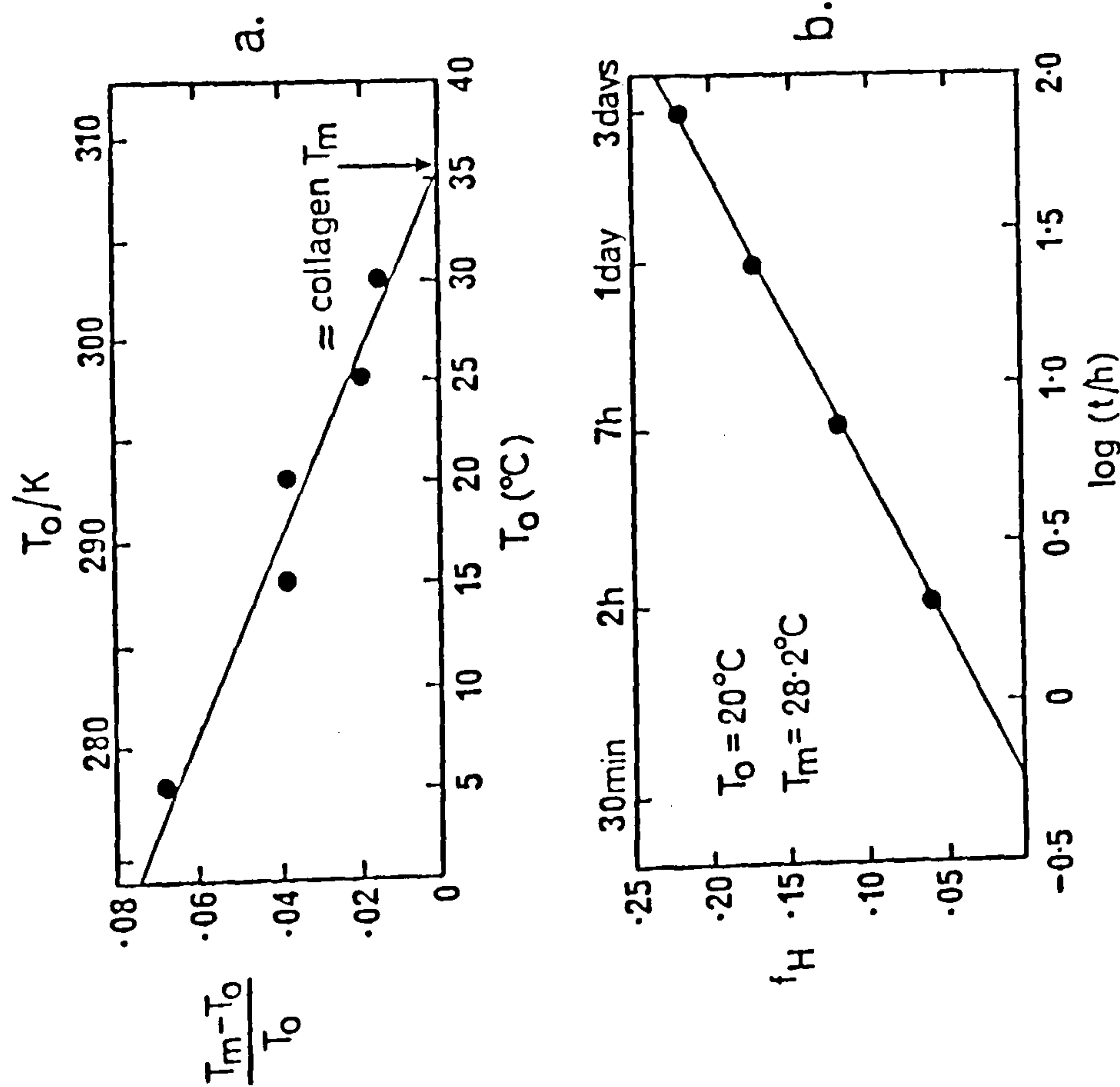


Figure 4. (a) Dependence of mid-point melting temperature ( $T_m$ ) on setting temperature ( $T_0$ ). (b) Increase in helix fraction ( $f_H$ ) with time ( $t$ ) at fixed temperature ( $T_0$ ), illustrated for  $T_0 = 20^\circ\text{C}$ .



# PROPOSED MODEL FOR GELATIN RENATURATION

To interpret this behaviour we propose the following set of 'minimum assumptions' illustrated schematically in Fig. 7.

- 1) Helix formation is initiated at a  $\beta$ -bend or similar metastable 'kink' which brings two chain segments into close spatial proximity.
- 2) These may then form a helix nucleus by collision with a third strand.
- 3) At low concentration this is most likely to be a segment of the same chain while at higher concentration an intermolecular collision is more probable.
- 4) Nucleation is rate-limiting, so that the intramolecular process shows first-order kinetics and the intermolecular process is second-order.
- 5) Subsequent helix growth is rapid, and proceeds as far as is geometrically possible (i.e. until reaching the end of one of the participating strands or until, as illustrated in Fig. 7, an intramolecular loop is fully utilised).

Qualitatively this model offers a simple explanation of the higher rigidity of gels whose thermal history has included a holding period at higher temperature. Since intramolecular helices involve three strands from the same chain their maximum possible length will be one third of the chainlength while for intermolecular helices, where only two of the participating strands are from the same chain, the maximum length will be half the chainlength. We would therefore expect the requirement for progressively longer helices with increasing temperature (6) to favour formation of intermolecular helices (which will of course contribute to the strength of the gel network) in preference to shorter, intramolecular structures (which are 'wasted').

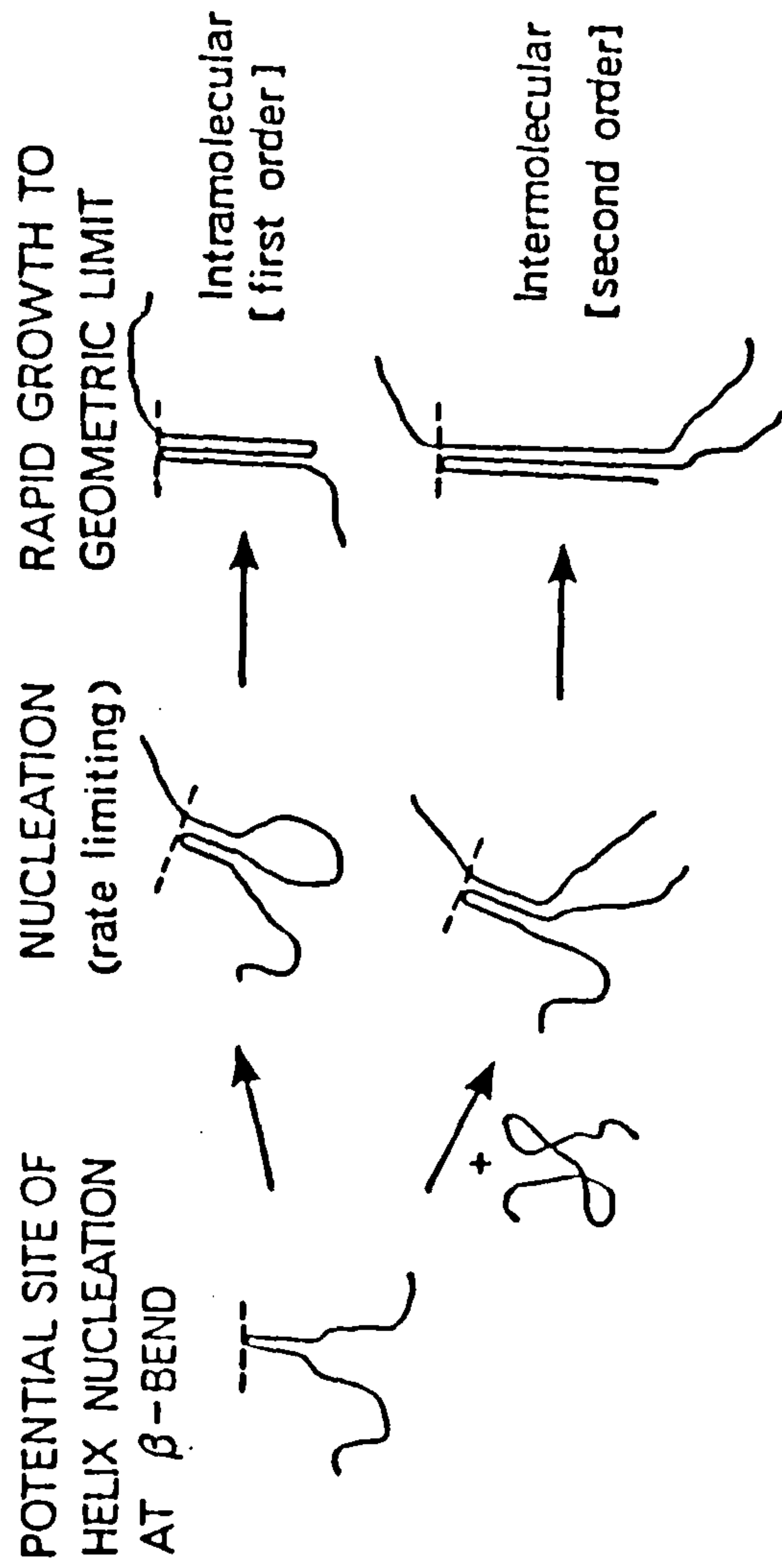


Figure 7. Schematic illustration of proposed kinetic model.

At concentrations below  $\sim 0.5 \text{ mg ml}^{-1}$  the initial slope ( $S = df_H/dt$ ) remains constant, as shown in Fig. 6; thus since  $f_H = [\text{helix}]/c$ , the absolute rate of helix formation is directly proportional to concentration ( $c$ ), and so the reaction displays first-order kinetics, consistent with an intramolecular process. At higher concentrations, however, the value of  $S$  increases substantially (by almost an order of magnitude over the concentration range studied), indicating the onset of a second mechanism of conformational ordering. To examine the kinetics of this second process we have subtracted the constant value of  $S$  at low concentrations ( $S_1$ ) from the experimental values of  $S$  at higher concentrations to obtain the contribution ( $S_2 = S - S_1$ ) from the second process. As shown in Fig. 6, the slope of  $\log S_2$  vs  $\log c$  is very close to unity ( $\sim 1.02$ ) and thus the additional process at higher concentration is second-order, consistent with the involvement of two chains in the rate-limiting step to triple-helix formation.

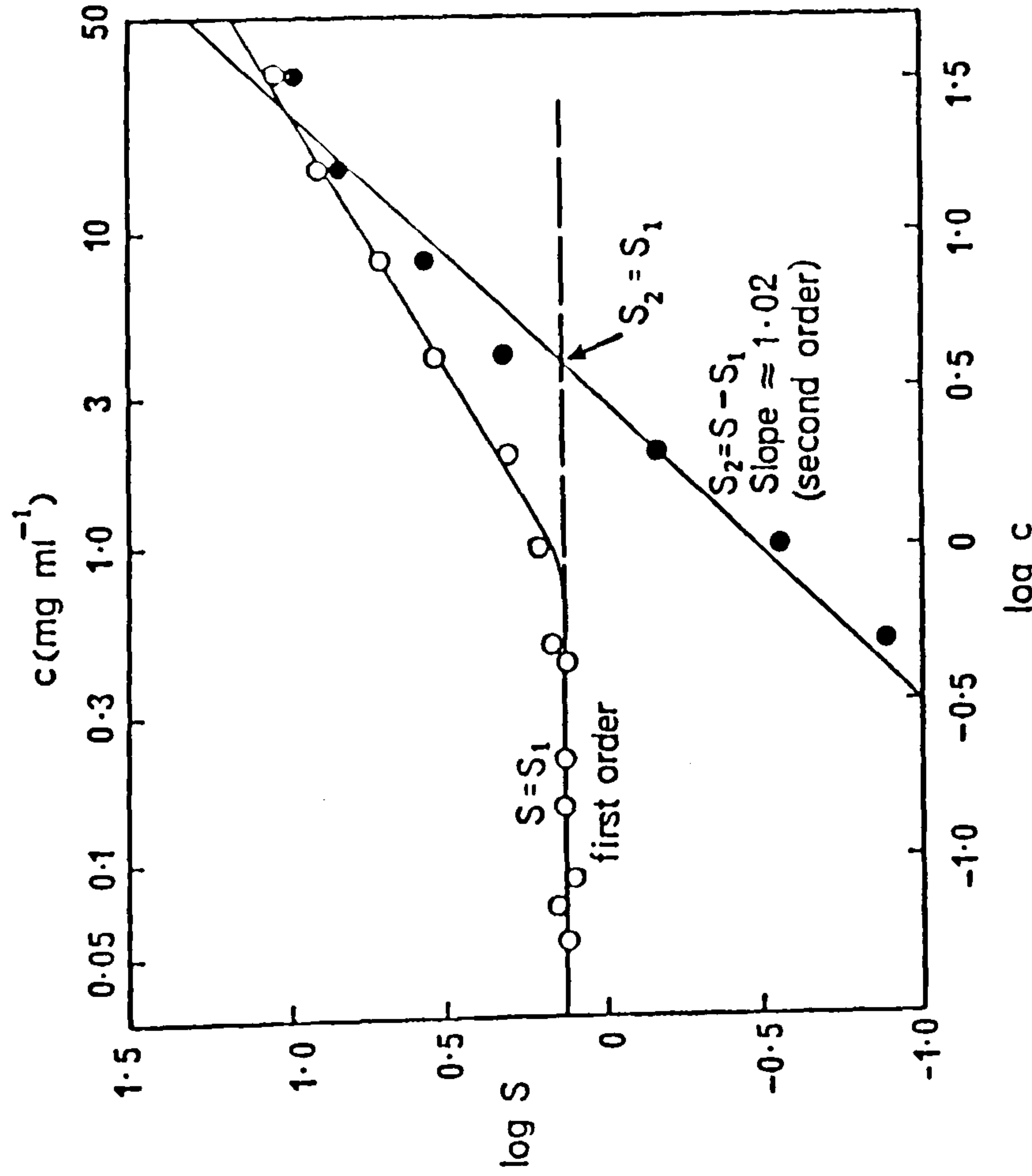


Figure 6. Concentration-dependence of 'initial slope' kinetics for renaturation of gelatin ( $10^\circ\text{C}$ ). Open symbols: observed slope ( $S = df_H/dt$ ); filled symbols:  $S_2 = S - S_1$  where  $S_1$  is the constant value of  $S$  at low concentration.



## MONTE-CARLO SIMULATION

As a first step in making this argument more quantitative we have carried out a preliminary computer simulation of the renaturation process based on the model proposed above. Briefly, we first generate a large population of chains; in the results reported here we have used 1000 chains with a gaussian length-distribution centred at 0.8 of the maximum length. The probability that helix formation is intermolecular rather than intramolecular  $[p(\text{inter})]$  is chosen to simulate concentration. In each cycle of the program a chain is selected at random (with the probability of selection directly proportional to the chain-length). If it is disordered (as it of course would be at the start of the simulation) then a point within it is chosen at random to simulate the position of the  $\beta$ -bend shown in Fig. 7. A second point is chosen at random, either within the same chain, to simulate intramolecular nucleation, or within a second, randomly selected disordered sequence, to simulate intermolecular nucleation; the intermolecular option is chosen if  $p(\text{inter})$  exceeds a random number generated between 0 and 1. In either case the second point is regarded as the position at which the third strand joins the  $\beta$ -bend to form the helix nucleus (Fig. 7), and the maximum potential helix length ( $L$ ) is calculated. The stability of the potential helix is then assessed by calculating the probability of unwinding,  $p(\text{un}) = A \exp(-BL)$ , where  $A$  and  $B$  have pre-selected values chosen to simulate temperature, as discussed later. If  $p(\text{un})$  exceeds a random number generated between 0 and 1, nucleation is considered to have failed and the program continues to the next cycle, leaving the chain(s) disordered. Otherwise the three participating strands of the helix are stored as ordered sequences, and any residual disordered loops or tails are considered as additional candidate sequences for helix formation in later cycles of the program.

So far we have considered the case where the sequence selected randomly at the beginning of the cycle is disordered. If, however, it is already ordered (which becomes progressively more likely as the simulation proceeds) then the stability test described above is applied. If  $p(\text{un})$  is less than the random number generated the program passes on to the next cycle leaving the helix intact; otherwise the chosen sequence and its two helix partners are considered to have become disordered and are combined with any contiguous disordered sequences in the same chain(s). The number and length-distribution of inter- and intramolecular helices may be printed out at any stage, and iteration continued with the same values of  $A$  and  $B$  (to simulate longer times at the same temperature) or with different values (to simulate temperature change).

In the present preliminary studies a combination of values for  $A$  and  $B$  were chosen by trial and error to give a reasonable quantitative match (Fig. 5) to experimental reaction progress curves at  $10^\circ\text{C}$  ( $A = 5$ ;  $B = 11.5$ );  $A$  was then held constant and  $B$  increased or decreased to simulate lower or higher temperatures respectively (i.e. as  $B$  increases the probability that a helix of a given length will unwind decreases). By comparison of the helix fraction attained after a large number of iterations (e.g. 10,000) with experimental values at long times (where in both

cases the rate of change in  $f_H$  is several orders of magnitude lower than the initial rate) we have chosen  $B = 75$  to simulate the final quench temperature in our DSC experiments ( $5^\circ\text{C}$ ), and  $B = 15$  to simulate a typical holding temperature ( $\sim 20^\circ\text{C}$ ).

Figure 8 shows the length-distribution of helical sequences obtained after 0, 1000 and 5000 cycles at,  $A = 5$ ,  $B = 15$  followed by iteration at  $A = 5$ ,  $B = 75$  until the overall helix fraction had attained an essentially constant value  $\sim 72\%$  in all cases). The results from this simulation parallel closely the experimental observations by optical rotation and DSC (Figs. 1 and 3). When direct cooling to  $5^\circ\text{C}$  is simulated (iteration at  $B = 75$  only) the distribution is monodisperse and centred at low helix length (i.e. low melting); prior iteration at  $B = 15$  (to simulate holding at  $20^\circ\text{C}$ ) shifts the populations of helix lengths to higher values (i.e. higher melting) and eventually leads to a bimodal distribution, with curves crossing at an isosbestic point as observed experimentally.

Although the relative probabilities of inter- and intramolecular helix formation were set to be equal ( $p(\text{inter}) = 0.5$ ) the proportion of intermolecular helices after iteration at  $B = 15$  (to simulate holding at  $20^\circ\text{C}$ ) was about  $75\%$  of the total, with a length-distribution centred at much higher values ( $\sim 32\%$  of the maximum chainlength, in comparison with  $\sim 18\%$  for intramolecular).

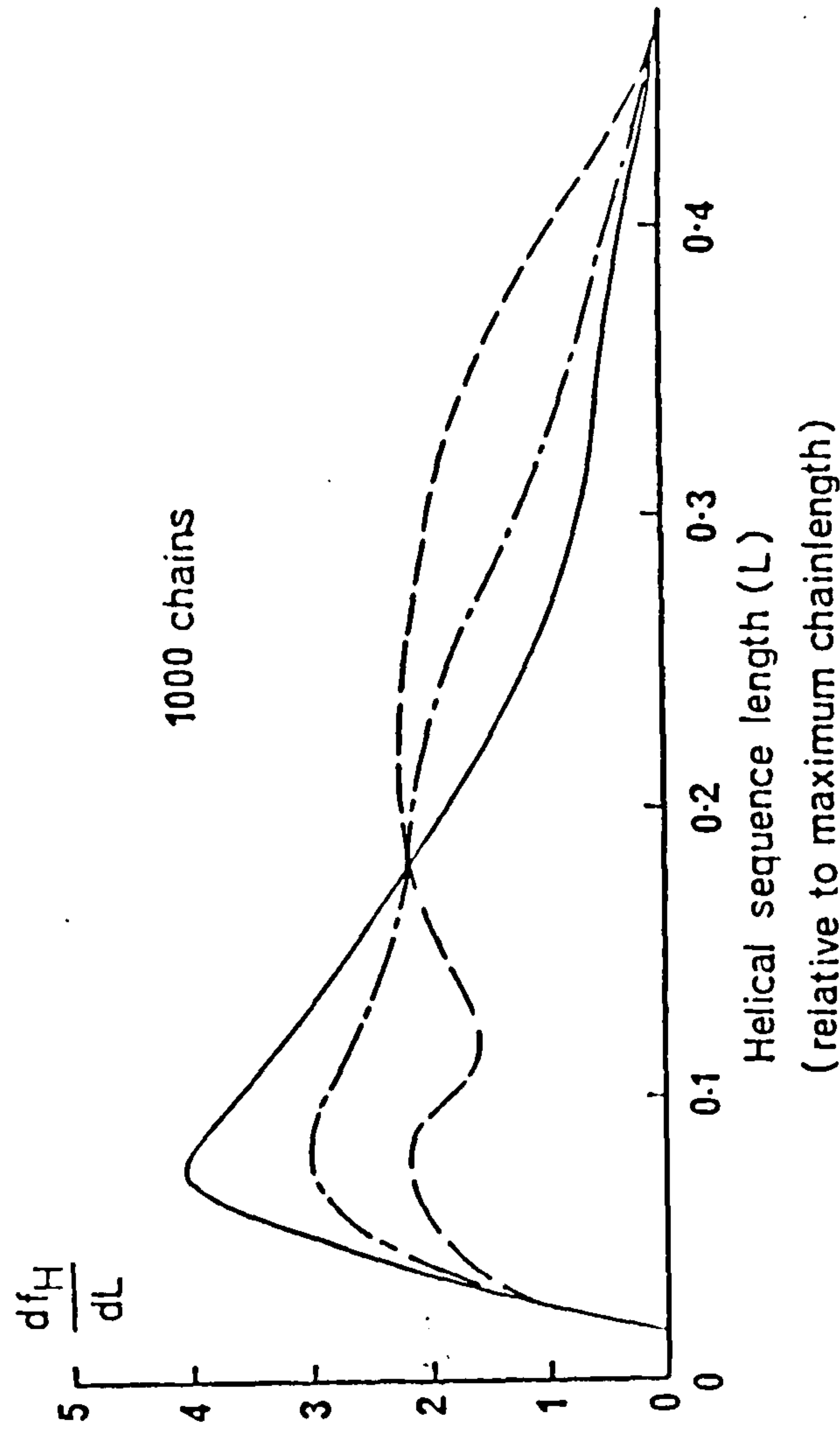


Figure 8. Monte-Carlo simulation of gelatin renaturation. Results are shown for the length-distribution of helical sequences after 0 (—), 1000 (---) and 5000 (-.-) cycles at  $A = 5$ ,  $B = 15$  followed by iteration to essentially constant helix fraction ( $f_H = 0.72$  in all cases) at  $A = 5$ ,  $B = 75$ , with  $p(\text{inter})$  set at 0.5 throughout.



On subsequent further iteration at  $B = 75$  (to simulate cooling to  $5^{\circ}\text{C}$ ) this population of long (i.e. high-melting) intermolecular helices remains virtually unchanged but is augmented by additional shorter helices (inter- and intramolecular), leading to the bimodal length-distribution shown in Fig. 8.

In summary, therefore, we propose that from simple geometric and statistical considerations intermolecular triple helices requiring a maximum of two strands from a single chain will have a considerably higher average length and therefore a greater probability of formation and survival, particularly at higher temperatures, than intramolecular helices where a single chain provides all three strands, and that this simple proposal provides a unified interpretation of both the greater thermal stability and higher rigidity of gels whose thermal history includes a holding period at higher temperature.

#### ACKNOWLEDGEMENTS

We thank Drs. S.B. Ross-Murphy, I.T. Norton and D.M. Goodall for helpful discussions, and the AFRC for the award of a Cooperative Research Studentship to S.M. Clegg.

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